STUDIES OF ACTION OF HEAVY METALS ON CAFFEINE DEGRADATION BY IMMOBILISED *LEIFSONIA* SP. STRAIN SIU

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ABSTRACT
Caffeine is an important naturally occurring compound that can be degraded by bacteria. Excessive caffeine consumption is known to have some adverse problems. Previously, Leifsonia sp. strain SIU capable of degrading caffeine was isolated from agricultural soil. The bacterium was tested for its ability to degrade caffeine as the sole carbon and nitrogen source. The isolate was encapsulated in gellan gum and its ability to degrade caffeine in the presence of heavy metals was determined. Out of the nine heavy metals tested, Copper (Cu), Mercury (Hg), and Silver (Ag) had significant effects on caffeine degradation at 1mg/L. Therefore, the concentration of these heavy metals was varied from 0 - 1 mg/L to see at what concentration each metal it has effect. Ag and Hg showed effect at 0.1 mg/L with caffeine degradation of 64.05 and 52.17% respectively, while Cu showed effect at 0.8 mg/L with caffeine degradation of 64.74%. These bacterium features make it an ultimate means for caffeine bioremediation. This is the first report of effect of heavy metals on caffeine degradation by immobilised Leifsonia sp. strain SIU.
Keywords: Caffeine, Degradation, Heavy metals, Immobilisation, Leifsonia sp.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a naturally occurring purine alkaloid that is present in many plant species such as tea leaves, cocoa, coffee seeds, yerba mate, colanut, and guarana (Ashihara and Crozier, 2001). Though prolonged caffeine consumption increases alertness and concentration by overcoming fatigue (Smith, 2002; Ibrahim *et al.,* 2014), research on this compound revealed many deleterious effects that it may have on the human body. Prolonged caffeine consumption not only leads to addiction and subsequent withdrawal effects like headaches, nausea and drowsiness (Nehlig, 1999), it also results in many clinical conditions like increase in blood pressure, osteoporosis and cardiac arrhythmia (Lorist and Tops, 2003; Nawrot *et al.,* 2003; Ibrahim *et al.,* 2015a).

Caffeine is one of the major toxic compounds generated by solid wastes in the tea and coffee industries, i.e., coffee pulp, husk and tea waste. In spite of the fact that these wastes are enriched with proteins and carbohydrates, they cannot be used as animal feed because of the presence of caffeine and other toxic compounds (Pandey *et al.,* 2000). Channeling of these wastes to nearby lakes and rivers would have effect on aquatic organisms (Ibrahim *et al.,* 2015b; Gibson *et al.,* 2009). As such, caffeine degradation is paramount in the health and environmental point of view.

Heavy metals are essential as trace elements or trace metals to all living organisms, but excessive concentrations can cause severe toxic effects to living organisms such as human, plants and animals (Poli *et al.,* 2009). The existence of heavy metals at the contamination area is the major limiting factor for bioremediation, as many organisms cannot withstand high concentration of heavy metals thus losing their capacity to degrade the contaminants. The wide spread occurrence of heavy metals in the environment can be due to disposal of waste products (Lasat, 2002). Moreover, it can also be due to such industries that involves metal processing, wood combustion, mineral and coal oil, and also from the use of plant protection (Schinner and Klauser, 2005). In Malaysia, heavy metals enter the environment either from agricultural and industrial activities or human waste (Alkarkhi *et al.,* 2008). Alkarkhi *et al.,* (2008) equally reported that heavy metals were detected in Jejawi and Juru River. In the report, Pb, Cd, As, and Hg contents exceeds the Malaysian standards level. In another report, Ramli and Ang, (2000) reported that ex-mining lands in Perak, and Bidor are contaminated with four heavy metals, Pb, Cd, As, and Hg. Besides that, Zn, Cu, Ni, and Fe were also detected in Kg. Sungai Berembang (Perlis), Pantai Teluk Air Tawar and Sungai Layar (Kedah) (Yap *et al.,* 2009). This shows that the metals are transported to marine environmental river and through estuaries. This research is aimed at studying the action of heavy metals on caffeine degradation by immobilised *Leifsonia* sp. strain - SIU.

Previously, we have isolated a new bacterium that is capable of utilising caffeine as a sole source of carbon and nitrogen and was identified as *Leifsonia* sp. strain SIU (Ibrahim *et al.,* 2015b). The bacterium was immobilised and tested on the effect of heavy metals. Out of all the heavy metals tested, it shows Cu, Hg, and Ag has effect on caffeine degradation. This study was aimed at showing at what concentration do these heavy metals have effect on caffeine degradation by immobilised *Leifsonia* sp. strain SIU.

MATERIALS AND METHODS
Chemicals
Caffeine anhydrous >99% was purchased from Sigma, Aldrich USA. Other chemicals used are of analytical grade that were obtained from recognised chemicals suppliers, Fisher (Malaysia) and Merck (Darmstadt, Germany).

Media
Previously isolated *Leifsonia* sp. strain SIU was cultured at 30 °C in sterilised caffeine liquid medium (CLM) containing the following (g/L): 0.4 K_2HPO_4, 0.2 KH_2PO_4, 0.1 NaCl, 0.1 $MgSO_4$, 0.01 $MnSO_4.H_2O$, 0.01 $Fe_2(SO_4)_3$, 0.01 $NaMoO_4.2H_2O$, 0.4 NH_4Cl. The media contains 0.3 g/L of caffeine in addition to the above compositions. Carbon sources if any added to the medium were sterilised separately and then mixed to the medium under aseptic conditions. For solid medium, (25 g/L) agar was added to the caffeine medium. The isolates were maintained and sub-cultured in the caffeine agar medium.

Cell Immobilisation Protocol
Cell immobilisation using gellan gum as a gelling component was carried out following the technique described by Ahmad *et al.,* (2012). Briefly, gellan gum (0.75% w/v) was added to 100 mL deionised water and heated to 75 °C. Then, $CaCl_2$ (0.06% w/v) was added to the mixture and slowly cooled to 45 °C. The pH of the solution was adjusted to pH 7.0 using 0.1 M NaOH. This phase is called Pregel solution. *Leifsonia* sp. strain SIU were centrifuged at 7000 rpm for 10 min at room temperature to obtain the pellet. Wet weight of the resulting bacterial pellet (3.50 g) was dispersed in the gum mixture and continuously stirred. Beads were formed by using a peristaltic pump by dropping the gum mixture through a modified pipette tip into sunflower-canola oil containing 0.15% Span 80 as an emulsifier. The uniformly-sized beads were then separated from the oil by transferring them into $CaCl_2$ 0.1% (w/v). After 2 h, the beads were repeatedly rinsed with Tween 80 solution 0.1% (v/v) to remove the oil phase from the microbeads. Prior to the usage, the beads were kept overnight in distilled water at 4 °C before being harvested by filtration.

Effect of Heavy Metals
The effect of some heavy metals on caffeine degradation by immobilised *Leifsonia* sp. strain SIU was studied. Heavy metals that have effect on caffeine degradation were picked and as such, the final concentration of these metals in the degradation media was varied from 0 - 1 mg/L. The reaction mixture was incubated in an optimised condition for 24 h in a rotary shaker at 150 rpm. The amount of caffeine degradation was measured using high performance liquid chromatography (HPLC). All the experiments were conducted in triplicates.

Analytical Methods
Degradation of caffeine was estimated by HPLC (Agilent 1100 series from Agilent technologies, Waldbronn, Germany, Product No. G2170AA) equipment using a ZORBAX® SB-C18 column (USA, Product No. 880975-902, Batch No. B03024) with 10 mM ammonium phosphate buffer (pH 2.5)/ acetonitrile (4:1, v/v) as mobile phase (with reference to specifications in the HPLC user manual). Pure caffeine at 2 mg/mL was used as the standard. Retention time of caffeine was found to be 2.1 min at a flow rate of 1 mL/min and at 30 °C. Detection of caffeine was done at 254 nm (detector sensitivity: 1×10^{-14} absorbance unit) (Ahmad *et al.,* 2015).

The percentage of caffeine degradation was calculated as follows.

Caffeine degradation (%) =

where *a* is the initial concentration and *b* is the residual caffeine concentration.

RESULTS AND DISCUSSION
Effect of Heavy Metals on Caffeine-degrading Activities by Immobilised Cells
It was been shown previously that many heavy metals can affect microorganisms even at low concentrations (Kim, 1985). Figure 1 shows the effect of different heavy metals on caffeine degradation by immobilised cells at 1 mg/L after 24 h of incubation. From the result obtained for immobilised cells, three out of nine heavy metals tested have inhibitory effect on caffeine-degrading activities. These include Cu, Hg and Ag ($p<0.05$). Ag gave the highest effect on caffeine degradation, where only 21.33% was degraded, followed by Hg (23.45%) and Cu (26.14%) respectively. Other heavy metals such as Cd, Zn, Ni, and Cr, showed significant difference on caffeine-degrading activities ($p<0.05$) with a degradation percentage of 97.74% 82.11%, 95.35%, and 96.64% respectively. More so, degradation of As and Pb showed that there were no significant difference on caffeine-degrading activities ($p>0.05$) with a degradation percentage of 92.16%, and 92.90% respectively. Medium without addition of heavy metals showed high caffeine degradation for immobilised cells compared to media with heavy metals.

Heavy metals affect degradation by inhibiting the bacterial growth (Kim, 1985) or the enzymes that play a role in degradation. Heavy metals also affect the membrane structure by disturbing the electron transport chain. Therefore, the presence of heavy metals on immobilised cells was shown to have negative effect on caffeine degradation.

Pollutants and toxic compounds degradation in the environment are mostly due microbial activities as shown in several reports for instance the bioremediation of heavy metals (Shukor *et al.,* 2010), phenol derivatives (Aguayo *et al.,* 2009; Seja´kova *et al.,* 2009), diesel (Sadouk *et al.,* 2009), azo dyes (Gonza´lez-Gutie´rrez *et al.,* 2009; Syed *et al.,* 2009; Revankar and Lele, 2006), and pesticides (Chatterjee *et al.,* 2010). However, the growth of these organisms is inhibited at high concentrations of the toxic compounds which turn to be very toxic, thus limiting the effectiveness of the caffeine biodegradation.

This result is in accordance with the result obtained by Hong *et al.,* (2007) where heavy metal has a slightly inhibitory effect on dibenzofuran. These toxic compounds also exert their toxic effects by inhibiting caffeine-degrading enzymes or other metabolic enzymes needed for growth (Nies, 1999).
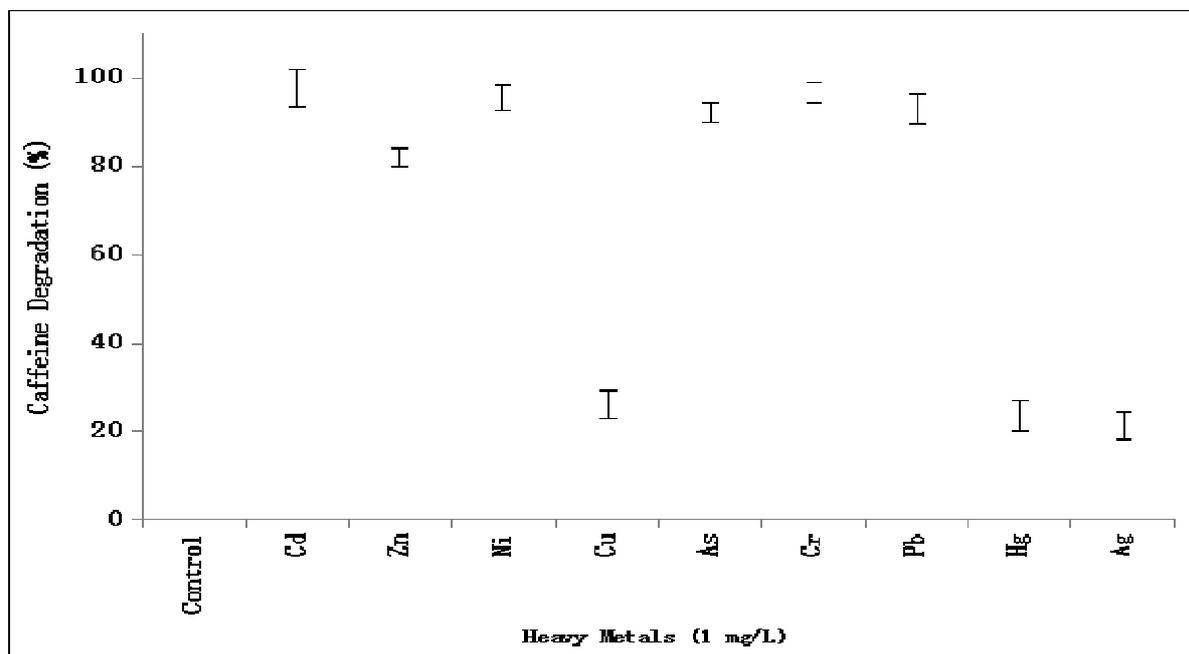

Figure 1. Effect of different heavy metals on caffeine degradation at initial concentration of 0.3 g/L by immobilised *Leifsonia* sp. strain SIU after 24 h of incubation time. Data represent mean ± STDEV, n=3.

Effect of Copper on Caffeine-degrading Activities by Immobilised Cells

Copper is an important cofactor in many of the enzymatic processes taking place within the cell. However, Cu is highly reactive and for that reason it is toxic to the cell in high concentrations and is natural anti-microbial materials (Abushelaibi, 2005). Ancient civilizations exploited the antimicrobial properties of copper long before the concept of microbes became understood in the nineteenth century (Dollwet and Sorenson, 1985). In addition to numerous copper medicinal preparations, it was also observed centuries ago that water contained in copper vessels or transported in copper conveyance systems was of better quality than water contained or transported in other materials. The antimicrobial properties of Cu are still under active study. Molecular mechanisms responsible for the antibacterial action of copper have been a subject of intensive research. Scientists are also actively indicating the intrinsic efficacies of Cu alloy touch surfaces to destroy a wide range of microorganisms that threaten public health.

In this study, the effect of Cu on caffeine degradation was determined from 0 to 1 mg/L. The results (Figure 2) obtained showed that Cu concentrations from 0.1 to 1 mg/L affected caffeine-degrading performance ($p > 0.05$). However, there was no caffeine degradation inhibition between 0 to 0.5 mg/L. Degradation was observed when the concentration exceeds 0.5 mg/L ($p < 0.05$). At 0.6, 0.7 and 0.8 mg/L the degradation was found to be 94.28, 92.27 and 64.73% respectively while at 0.9 and 1 mg/L it shows the highest inhibition of 33.95 and 26.25% respectively. Cu has antimicrobial effects that can inhibit bacterial growth and degradation.
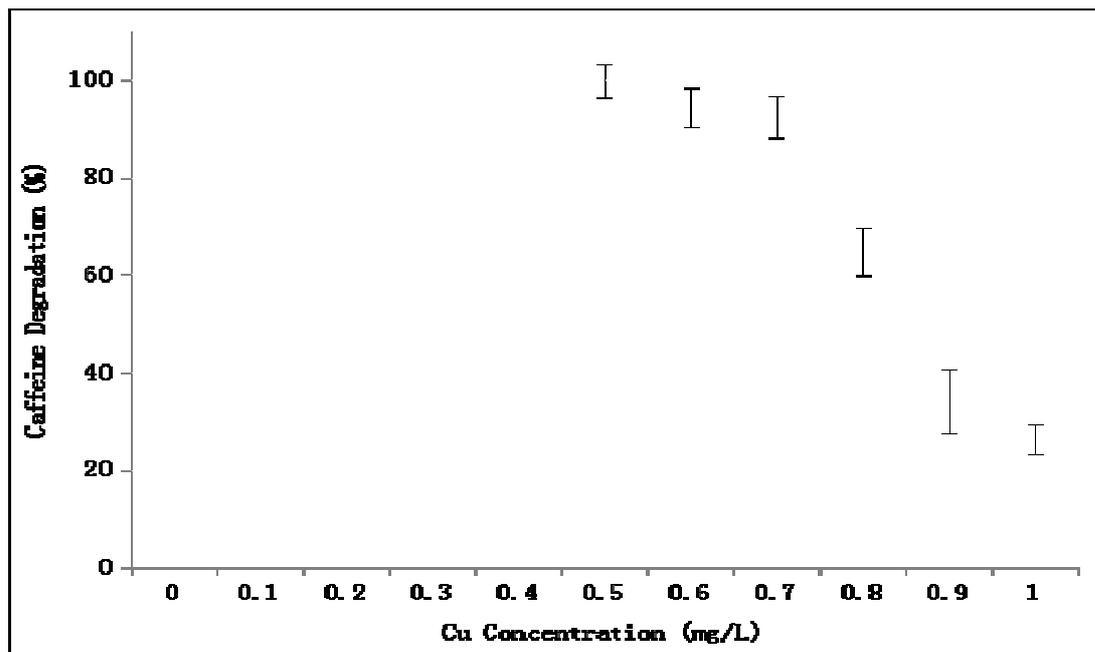
Figure 2. Effect of Cu on caffeine degradation of immobilised *Leifsonia* sp. strain SIU. Data represents mean ± STDEV, n=3.

Effect of Silver on Caffeine-degrading Activities by Immobilised Cells

Silver (Ag) is used as an antibacterial agent in medical practices as well as in textile fabrics (Atiyeeh *et al.,* 2007). Ag is found relatively high in concentration in the sludge sample (Sethu *et al.,* 2005), has the potential to prevent bacterial colonisation by its broad-spectrum of antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, protozoa, and some viruses (Tien *et al.,* 2008). According to Malaysian DOE 2004 report, the exported wastes were derived from 36 waste generators and comprised of metal hydroxide sludge containing heavy metals such as Ag, Cu, and Ni (DOE, 2004). Effect of Ag on caffeine degradation was studied.

In this study, the effect of Ag on caffeine degradation was determined from 0 to 1 mg/L. The result (Figure 3) obtained showed that Ag concentrations from 0.1 to 1 mg/L affected caffeine-degrading performance ($p < 0.05$). Ag can be considered as harmful metal to immobilised *Leifsonia* sp. strain SIU due to its effects on caffeine degradation at a very low concentration. At 0.1 mg/L caffeine degradation was 64% and 30% at 1.0 mg/L respectively. It illustrates that Ag can simply entered immobilised bead due to it high ionisation energy and bead faster than caffeine substance (Milton and Reetha, 2012).
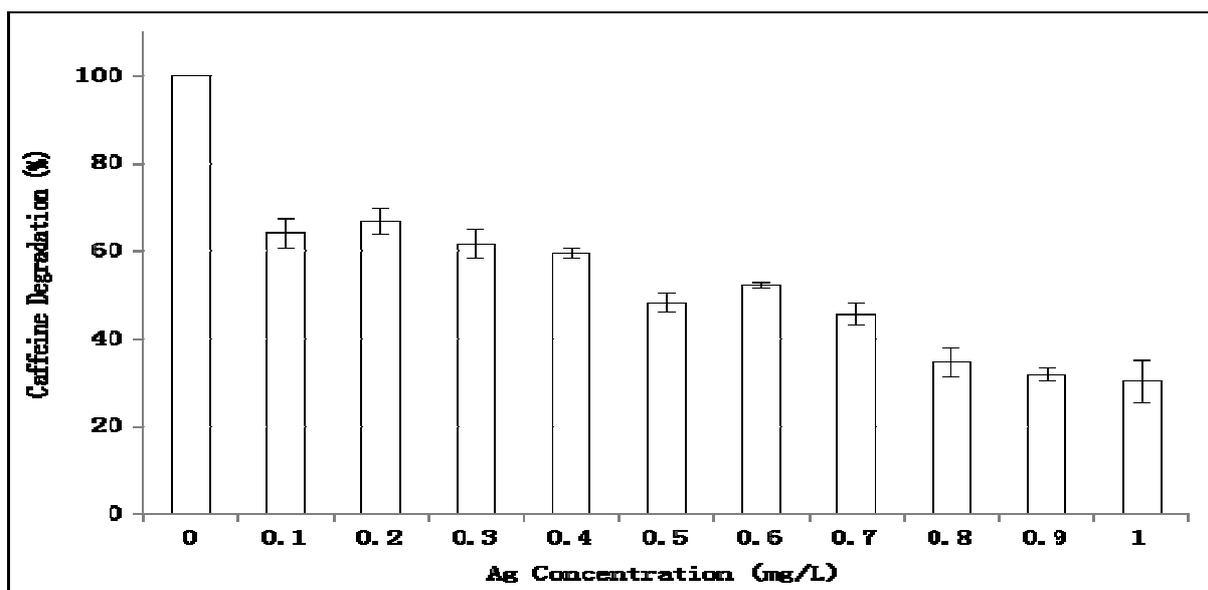

Figure 3. Effect of Ag on caffeine degradation of immobilised *Leifsonia* sp. strain SIU. Data represents mean ± STDEV, n=3.

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Effect of Mercury on Caffeine-degrading Activities by Immobilised Cells

Mercury (Hg) is considered the most toxic non-radioactive metal in the environment and toxic in any form (Abdel-Salam *et al.,* 2010). Asia is one of the major contributors of anthropogenic atmospheric Hg and serious Hg pollutions were found in the area influenced by chemical industry, gold mining and Hg mining (Li *et al.,* 2009). In Malaysia, the main source of Hg in rivers are from urban and agricultural run-off and effluent from industries sewage treatment plants, domestics discharges, constructions, earthwork and pig farms (DOE, 2002). It appears that, this heavy metal also appears in caffeine-contaminated site. Hence, effect of Hg on caffeine degradation was studied.

In this study, the effect of Hg on caffeine degradation was determined from 0 to 1 mg/L. The results (Figure 4) obtained showed that Hg concentrations from 0.1 to 1 mg/L affected caffeine-degrading performance. Increasing Hg concentration to 0.4 mg/L leads to decrease in caffeine degradation by 27%, which can be classified as detrimental. Radjendirane *et al.,* (1991) reported that Hg could also inhibit hydroxylases activity like aryl 2,4-dichlorophenol hydroxylase and 3-hydroxybenzoate-6-hydroxylase. Analysis of variance showed that there was no significant difference in caffeine degradation between 0.4, 0.5 0.6 and 0.7 mg/L ($p>0.05$) but the remaining concentration shows there was a significant difference between them ($p<0.05$).
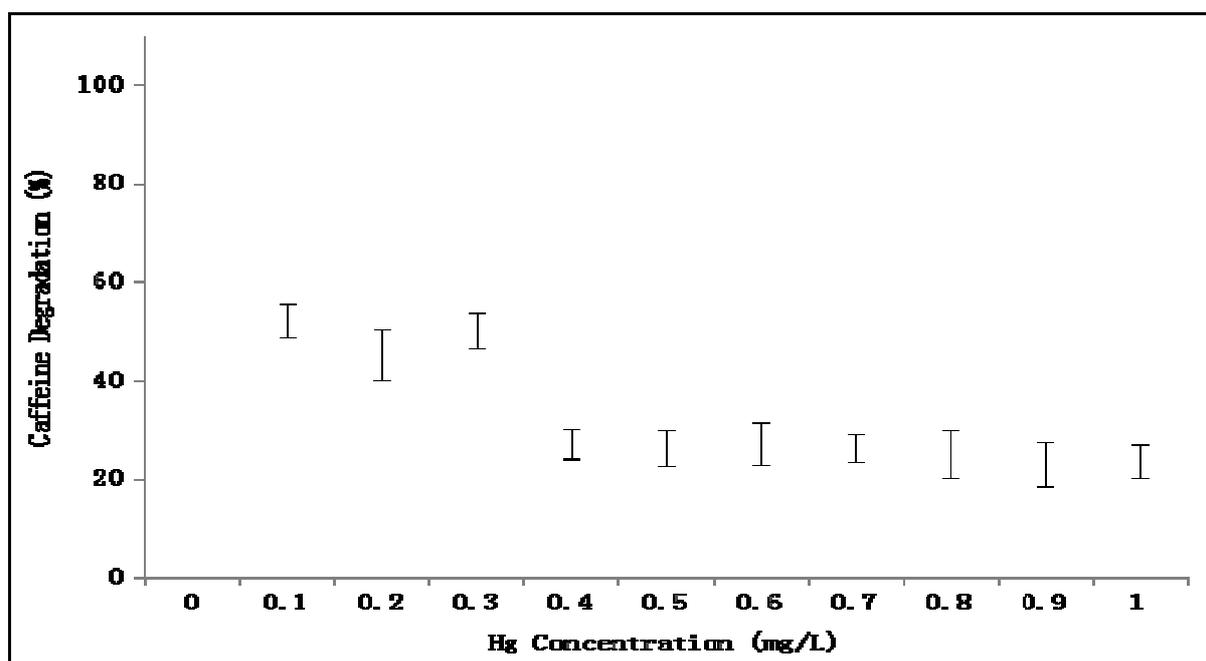

Figure 4. Effect of Hg on caffeine degradation of immobilised *Leifsonia* sp. strain SIU. Data represents mean ± STDEV, n=3.

Heavy metals are toxic to most microorganisms at specific concentrations and often cause serious upsets in biological waste treatment plants. The mechanism by which these heavy metals affect the microorganisms to degrade caffeine is not clear. Nevertheless, it has been suggested that heavy metals block the enzyme systems or interfere with some essential cellular metabolite of bacteria (Majid, 2010). Heavy metals inhibit microorganisms by blocking essential functional groups or interfering with essential metal ions incorporation of biological molecules. In some cases microorganisms are resistant to some heavy metals through different possible mechanisms (Martins *et al.,* 2008).

The inhibition of caffeine demethylase enzyme for caffeine degradation follows non-competitive inhibition model, which is an inhibition where by the inhibitor (heavy metals) forms complex at a point other than the active site, as such it alters the structure of the enzyme in such a way that the substrate may get attached to the active site but products are not

formed. The reaction goes on decreasing as more and more inhibitors contact the enzyme till saturation is reached.

Conclusion

The effect of heavy metals on caffeine biodegradation was studied using a newly isolated bacterium immobilised *Leifsonia* sp. strain SIU. The isolate was encapsulated in gellan gum and its ability to degrade caffeine in the presence of heavy metals was determined. Out of the nine heavy metals tested, Copper (Cu), Mercury (Hg), and Silver (Ag) have effects on caffeine degradation using 1 mg/L of the heavy metals. Ag and Hg showed significant effect at 0.1 mg/L with caffeine degradation of 64.05 and 52.17% respectively, while Cu showed effect at 0.8 mg/L with caffeine degradation of 64.74%. The present information regarding the effect of heavy metals on caffeine degradation as an effective degrader can be useful to the real state where degradation is essential in caffeine contaminated areas.

Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

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