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Enhanced caffeine degradation by immobilised cells of *Leifsonia* sp. strain SIU

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In a previous study, we isolated *Leifsonia* sp. strain SIU, a new bacterium from agricultured soil. The bacterium was tested for its ability to degrade caffeine. The isolate was encapsulated in gellan gum and its ability to degrade caffeine was compared with the free cells. The optimal caffeine degradation was attained at a gellan gum concentration of 0.75% (w/v), a bead size of 4 mm diameter, and 250 beads per 100 mL of medium. At a caffeine concentration of 0.1 g/L, immobilised cells of the strain SIU degraded caffeine within 9 h, which is faster when compared to the case of free cells, in which it took 12 h to degrade. The immobilised cells degraded caffeine completely within 39 and 78 h at 0.5 and 1.0 g/L, while the free cells took 72 and 148 h at 0.5 and 1.0 g/L, respectively. At higher caffeine concentrations, immobilised cells exhibited a higher caffeine degradation rate. At concentrations of 1.5 and 2.0 g/L, caffeine-degrading activities of both immobilised and free cells were inhibited. The immobilised cells showed no loss in caffeine-degrading activity after being used repeatedly for nine 24-h cycles. The effect of heavy metals on immobilised cells was also tested. This study showed an increase in caffeine degradation efficiency when the cells were encapsulated in gellan gum.

Key Words: caffeine; degradation; gellan gum; immobilisation; *Leifsonia* sp.

Introduction

The purine alkaloid caffeine (1,3,7-trimethylxanthine) is found in more than 60 plant species, with significant levels in coffee beans, tea, cocoa, etc. (Mazzafera, 1991; Suzuki et al., 1992). Caffeine has been found to account for the stimulatory action of coffee. It was used as a cardiotonic until the end of the 19th century, due to its stimulatory effects (Wijhi, 2002). Prolonged caffeine consumption has a deleterious effect on cardiac patients and can also lead to osteoporosis, malfunction of the foetus, cardiac arrhythmias, irregular muscular activity, adrenal stimulation, apathy, headache, and fatigue (Lorist and Tops, 2003; Smith, 2002). Apart from such negative aspects, decaffeination is essential from an environmental point of view. Liquid and solid wastes generated by tea- and coffee-processing industries contain high concentrations of caffeine. Channelling these wastes into rivers and lakes have negative effects on marine organisms (Gibson et al., 2009; Ibrahim et al., 2015b; White and Rasmussen, 1998). Even though these wastes are enrich with macromolecules such as proteins and carbohydrates, they cannot be used to feed animals due to the presence of toxic compounds such as caffeine, theobromine and theophylline (Ahmad et al., 2015; Ibrahim et al., 2015b; Mazzafera, 2002; Pandey et al., 2000).

Conventional methods of caffeine degradation such as supercritical carbon dioxide, water and solvent extraction methods have been used to degrade caffeine (Kim et al., 2008). These methods are non-specific, toxic, and costly. Microbial techniques of caffeine degradation are required

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to overcome these problems (Gokulakrishnan et al., 2005). Microbial caffeine degradation using free cells remains, as yet, a difficult challenge and requires a lot of research. For instance, it is difficult to isolate a strain that can tolerate and degrade a high concentration of caffeine within a short period of time (Gummadi et al., 2009; Ibrahim et al., 2014). In addition, the use of microorganisms such as bacteria, algae, fungi, and yeasts in free cells has been found to be inadequate for toxic compound degradation due to possible clogging (Godjevargova et al., 2004; Lakshmi and Das, 2013). The process of immobilised microbial cells can improve biodecaffeination efficacy and has many advantages over free-suspended cells for the biodegradation of xenobiotics (Baskaran and Nemati, 2006; Wang et al., 1997). Moreover, unlike free cells, immobilised cells are less likely to be affected by parasites, toxins, and predators (Prabua and Thatheyus, 2007).

The effectiveness of caffeine biodegradation can be improved by using cell immobilisation (Ahmad et al., 2012). The most commonly used immobilisation matrices for caffeine degradation are polyacrylamide, sodium-alginate, calcium-alginate agarose and agar, which is mainly because the procedures are simple, mild, and not toxic to the cells (Gummadi et al., 2009; Lakshmi and Das, 2013). However, the material is susceptible to degradation and has a low mechanical stability (Ha et al., 2009). Gellan gum is another commonly used matrix as the gel is stable over a wide pH range of 2–10, recommended in fermentation technology and non-toxic due to its mechanical and thermal stability (Ahmad et al., 2012; Ashtaputre and Shah, 1995; Camelin et al., 1993; Ha et al., 2009; Moslemy et al., 2002, 2003; Norton and Lacroix, 1990; Waller et al., 1986).

The growth of many microorganisms is inhibited at high concentrations of caffeine (Waller et al., 1986). However, the efficiency of the caffeine degradation could be further enhanced by the process of cell immobilisation (Vegesna, 2007), as immobilised cells are more stable than free cells. Immobilised cells are being widely used as they help to increase the efficiency of bioremediation (Duran and Esposito, 2000).

To date, this is the first report that describes the caffeine-degrading ability of immobilised cells of *Leifsonia* sp. To achieve a better degradation of an elevated concentration of caffeine, *Leifsonia* sp. strain SIU was encapsulated in gellan gum. Gellan gum is more stable and vigorous when compared with other natural polymers, such as calcium alginate (Moslemy et al., 2002; Wang et al., 2007). Furthermore, gellan gum has been recommended to be used in fermentation due to its non-toxicity and stability properties (Ahmad et al., 2012; Norton and Lacroix, 1990). Thus, the optimum gellan gum concentration is important as it affects the pore size and mechanical strength of the beads. As is intrinsic with most gel types such as alginate, agarose, and polyacrylamide gels, the pore size is proportional to the gel concentration. Moreover, pore size affects the diffusion of substrates and leakage of cells from the beads (Ahmad et al., 2012).

As reported previously, we have isolated a new aerobic bacterium from agricultural soil capable of utilising caffeine as sole carbon and nitrogen sources. The bacterium was identified as *Leifsonia* sp. strain SIU (Ibrahim et al., 2015b). In this study, *Leifsonia* sp. strain SIU was immobilised on gellan gum and its production was optimised. The capability of immobilised cells to degrade caffeine was verified and compared with the free cells. The degradation rates were also modelled.

Materials and Methods

Chemicals and media. Caffeine anhydrous >99% was purchased from Sigma, Aldrich USA. Other chemicals used are analytical grade that were obtained from Fisher (Malaysia) and Merck (Darmstadt, Germany). *Leifsonia* sp. strain SIU previously isolated from agricultural soil (Ibrahim et al., 2015b), was cultured at 30°C in a 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 $MnSO_4 \cdot H_2O$, 0.01 $Fe_2(SO_4)_3$, 0.01 $NaMoO_4 \cdot 2H_2O$, 0.4 NH_4Cl. The media contains 0.3 g/L of caffeine in addition to the above compositions. Carbon sources, if any, were added to the medium were sterilised separately and then added to the medium under aseptic conditions. For a solid medium, agar was added to the caffeine medium at a concentration of 25 g/L. The isolates were maintained and sub-cultured in the caffeine agar medium.

Analytical methods. The concentration of caffeine was estimated by High Performance Liquid Chromatography (HPLC; Agilent 1100 series, Waldbronn, Germany, Product No. G2170AA) equipped with a ZORBAX® SB-C18 column. A 10 mM ammonium phosphate buffer (pH 2.5)/acetonitrile (4:1, v/v) was used as mobile phase (with reference to specifications in the HPLC user manual). Pure caffeine at 2 mg/mL was used as the standard. The retention time of caffeine was found to be 2.1 min at a flow rate of 1 mL/min and at 30°C. The detection of caffeine was carried out at 254 nm (detector sensitivity: 1×10^{-14} absorbance unit) (Ahmad et al., 2015; Ibrahim et al., 2015a).

The percentage of caffeine degradation was calculated as follows:

$$\text{caffeine degradation } (\%) = \frac{(a-b)}{a} \times 100\%,$$

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

Gellan gum protocol. Cell immobilisation in gellan gum was carried out essentially following the technique described by Ahmad et al. (2012). In 100-mL deionised water, 0.75% (w/v) of gellan gum was originally added and heated to 75°C to completely dissolve the gellan gum. $CaCl_2$ (0.06% w/v) was then added to the mixture which was slowly cooled to 45°C. The pH of the solution was adjusted to 7.0 using 0.1 M NaOH. This phase is called the Pregel solution. About 3.50 g wet weight of the resulting bacterial pellet was dispersed in the gum mixture and continuously stirred. Beads were formed by using a peristaltic pump and 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

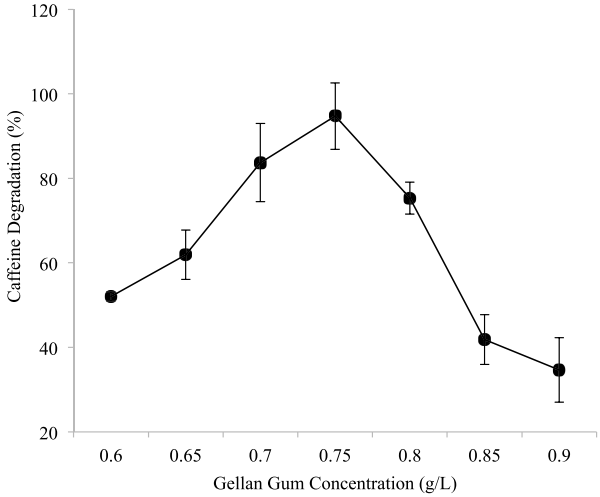

Fig. 1. Effect of gellan gum concentration on caffeine degradation by immobilised *Leifsonia* sp. strain SIU.

Data represents mean ± STDEV, $n = 3$.

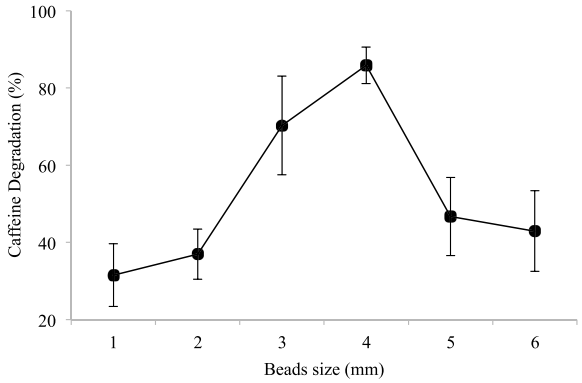

Fig. 2. Effect of bead size on caffeine degradation by immobilised *Leifsonia* sp. strain SIU.

Data represents mean ± STDEV, $n = 3$.

beads were then separated from the oil by transferring them into 500 mL of 0.1% (w/v) $CaCl_2$. After 2 h, the beads were repeatedly rinsed with 0.1% (v/v) Tween 80 solution to remove the oil phase from the microbeads. Before the beads were used for the caffeine degradation experiment, the beads were kept overnight in distilled water at 4°C before being harvested by filtration.

Optimisation of gellan gum condition. Three parameters were optimised for the degradation of caffeine by the immobilised bacteria. Initially, to determine the optimum gellan gum concentration, seven concentrations ranging from 0.6 to 0.9% (w/v) of gellan gum were used. Then, for optimisation of the bead size, 0.75% (w/v) gellan gum was used to prepare beads ranging in sizes of 1, 2, 3, 4, 5, and 6 mm diameter, and lastly, for the determination of the optimum initial cell loading, different quantities of beads ranging from 100 to 350 beads were used. The optimisation experiments were conducted in 100 mL caffeine media containing 0.3 g/L caffeine. The working solution was incubated for 24 h on an orbital shaker (150 rpm) and the caffeine concentration in each flask was measured using HPLC.

Caffeine degradation by immobilised and free cells. Comparison of the caffeine degradation by immobilised and free cells of *Leifsonia* sp. strain SIU was performed using the same initial biomass concentration for both the immobilised and free cells. The optimum number of beads was used to calculate the amount of microbial cells needed for the caffeine degradation using free cells. About 4% of *Leifsonia* sp. strain SIU was used in 100 mL caffeine medium, which was equivalent to 250 beads per 100 mL caffeine medium. Caffeine concentrations ranging from 0.1 to 2.0 g/L were tested for both the immobilised and free cell systems. Immobilised and free cells were added to 100 mL of caffeine media at the different caffeine concentrations in separate conical flasks and incubated in a rotary shaker at 150 rpm at room temperature. One millilitre of the samples was taken during some intervals and tested for caffeine degradation until complete degradation of caffeine was achieved. Caffeine media without bacteria were used as the controls. All the experiments were carried out in triplicate.

Reusability of immobilised cells. About 250 gellan gum beads were added to 100 mL of caffeine liquid media containing 0.3 g/L caffeine. The immobilised cells were incubated on an orbital shaker at 150 rpm for 24 h and the residual caffeine was measured during this period. After 24 h incubation, the medium was discarded and the beads were thoroughly washed and rinsed with distilled water before they were put into new fresh caffeine medium. The steps were repeated at 24-h cycles until a decrease in the caffeine degradation ability of the immobilised cells was observed.

Statistical analysis. All the experiments were carried out in triplicate. The data were analysed using Graph-Pad Prism version 3.0 and Graph-Pad InStat version 3.05. The data shown in the corresponding figures are the mean values of the experiment and expressed as mean ± standard deviation. Comparison between groups was performed using one-way analysis of variance (ANOVA) with post-hoc analysis by Turkey's test (Miller and Miller, 2002). $p < 0.05$ was considered statistically significant (Shukor et al., 2010).

Results and Discussion

Effect of gellan gum concentration

The effect of an appropriate gellan gum concentration is very important as it determines the mechanical strength of the beads as well as the effectiveness of the beads to degrade caffeine. This is because different gellan gum concentrations produce beads with different pore sizes. The rigidity and stability of the beads are also affected by the composition of the beads. Smaller pores only limit the diffusion of certain molecules, while very large pores may cause leakage (Chibata et al., 1987). Figure 1 shows the effect of the gellan gum concentration on caffeine degradation by immobilised *Leifsonia* sp. strain SIU. At a gellan gum concentration below 0.7% (w/v), caffeine degradation was observed to be 52.05 and 61.91% respectively, while at a concentration above 0.85% (w/v) the degradation was found to be very low (34.67%), and between a

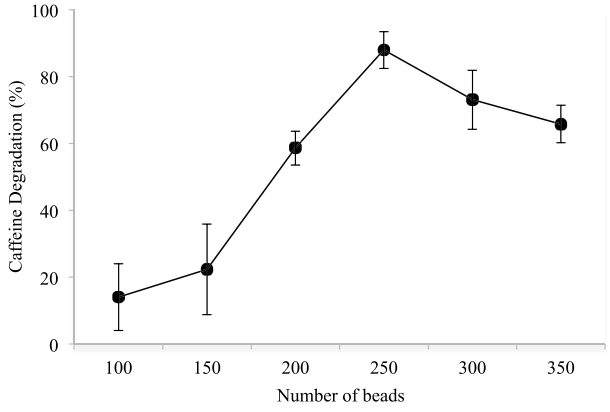

Fig. 3. Effect of gellan gum number of beads on caffeine degradation by immobilised *Leifsonia* sp. strain SIU.

Data represents mean ± STDEV, $n = 3$.

gellan gum concentration of 0.7 and 0.75%, caffeine degradation was found to be the highest. The optimum gellan gum concentration for caffeine degradation by the immobilised strain SIU was 0.75% (w/v). This is in compliance with the result obtained by Ahmad et al. (2012). Thus, subsequent experiments were carried out using a gellan gum concentration of 0.75% (w/v).

Effect of bead size

The optimum bead size can also improve the rate of caffeine degradation. The effect of bead size on caffeine degradation by the immobilised *Leifsonia* sp. strain SIU on 0.3 g/L caffeine was studied by measuring the caffeine degradation over a range of gellan gum bead sizes from 1 to 6 mm diameter at 0.75% (w/v) gellan gum concentration and the number of beads was maintained at 300 beads per 100 mL of caffeine medium. Small-sized beads had a big surface area (Beshay, 2003). By increasing the bead size, the cell mass encapsulated in the beads was also increased (Ahmad, 2008). Figure 2 shows the results for bead size, and the highest caffeine degradation rate was achieved with 4 mm beads compared with the other bead sizes. As such, nutrients and metabolites can diffuse through the semi-permeable membrane easily (Kailasapathy, 2002). Caffeine-degrading activity was markedly reduced with larger bead sizes tested. In view of the fact that the bacterial amount was kept constant during the preparation of the beads, the bead size is indicative of the cell density of the immobilised bacterial cells. The results revealed that caffeine degradation was reduced at higher cell densities.

Effect of initial cell loading (ICL)

The effects of initial cell loading (based on the number of beads) on caffeine degradation by immobilised *Leifsonia* sp. strain SIU was studied using different amounts (quantitative) of beads ranging from 100 to 350 beads in a 100 mL caffeine liquid media containing 0.3 g/L caffeine, with a gellan gum concentration of 0.75% (w/v), and a bead size of 4 mm. Figure 3 shows that a lower number of beads results in a lower caffeine degradation. This is because a low bead number generates unproductive regions and influences the particle density, as the quantity of the bacteria is not enough, thus lowering the total surface area of the immobilised beads (Beshay, 2003). An initial cell loading of 100 and 150 beads gave the lowest degradation compared with others ($p < 0.05$). The optimum number of beads for caffeine degradation by immobilised *Leifsonia* sp. strain SIU was between 250 and 300. A one-way ANOVA test result showed that there was a significant difference in caffeine degradation between 250 and 300 beads ($p < 0.05$).

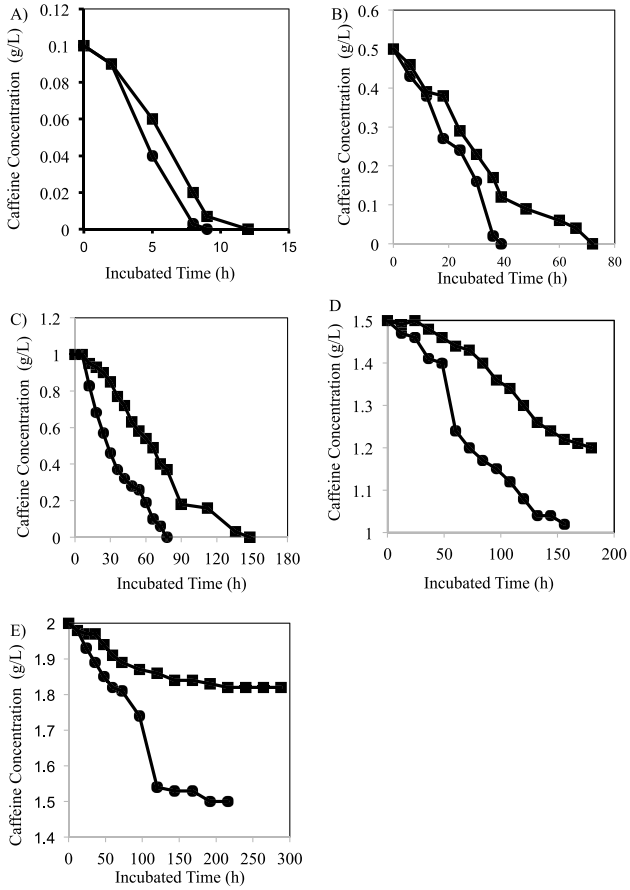

Fig. 4. Effects of different caffeine concentrations on the caffeine degradation over time of incubation of (filled square) free and (filled circle) immobilised *Leifsonia* sp. strain SIU.

Caffeine concentrations selected were; A) 0.1 g/L, B) 0.5 g/L, C) 1.0 g/L, D) 1.5 g/L and E) 2.0g/L. Data represents mean ± STDEV, $n = 3$.

It is generally expected that a higher number of beads results in a higher degradation. However, from the results, it is shown that both a higher and a lower number of beads compared with the optimum number has a negative effect on the caffeine degradation rate. One of the major problems in cell immobilisation is the rate of oxygen and nutrient diffusion via the gel matrix (Doherty et al., 1995). A higher cell density leads to a greater demand for nutrients and oxygen, which results in a reduced degradation rate of caffeine. At the optimum level of the initial cell loading together with the optimum bead size and gellan gum concentration, there is no restriction of nutrients and oxygen. With all these optimum conditions, the environment that surrounds the beads, such as the liquid layer, could easily aid in the movement of nutrients and oxygen into the cells. On the other hand, oxygen will be consumed faster than the rate it diffuses when the bead size and ini-

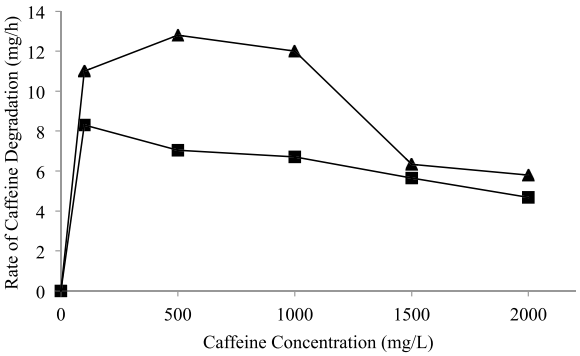

Fig. 5. Effect of caffeine concentration on degradation rate of immobilised and free *Leifsonia* sp. strain SIU.

Immobilised cells (filled triangle); free cells (filled rectangle).

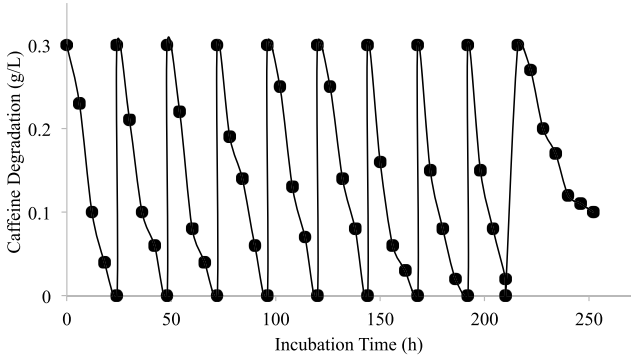

Fig. 6. Repeated usage of immobilised *Leifsonia* sp. strain SIU with an initial caffeine concentration of 0.3 g/L.

Data shown is for the repeated cycles only, with each cycle consisting 24 h. Data represents mean ± STDEV, $n = 3$.

tial cell loading per flask are increased. In these conditions, the struggle for oxygen diffusion by the cells increases and nutrient limitation also occurs as the nutrient/beads ratio decreases (Beshay, 2003; Gosmann and Rehm, 1986).

Comparison of caffeine-degrading activities of immobilised and freely-suspended cells

This study was conducted in order to compare caffeine degradation between immobilised and free cells of *Leifsonia* sp. strain SIU. The aim of this study is to observe the efficiency of the degradation between both types of cells. To determine the effect of caffeine concentration on caffeine degradation of immobilised and free cells, the cells were grown in a caffeine liquid media (pH 6.5) at 30°C containing initial concentrations ranging from 0.1 to 2.0 g/L. As a negative variable control, empty beads (without bacteria) were used at the same caffeine concentrations. There was no degradation recorded in the negative control beads at the end of the experiment. At 0.1 g/L caffeine (Fig. 4a), immobilised cells of strain SIU can degrade caffeine within 9 h when compared with free cells, which took 12 h to degrade caffeine. As the caffeine concentration was increased from 0.5 to 2.0 g/L, the rate of degradation also increased. Free cells showed a lower caffeine-degrading activity than the immobilised cells. Immobilised cells degraded caffeine completely within 39 and 78 h at 0.5 and 1.0 g/L, respectively, while the free cells took 72 and 148 h at 0.5 and 1.0 g/L, respectively to completely degrade caffeine (Figs. 4b and c). At concentrations of 1.5 and 2.0 g/L (Figs. 4d and e), the caffeine-degrading activity of both immobilised and free cells were inhibited. The strain was no longer able to degrade caffeine completely at these levels. At 1.5 g/L, caffeine degradation on immobilised cells was only 32.09 and 20.17% for free cells after 156 and 180 h, while at 2.0 g/L, the degradation was only 24.99 and 8.73% after 216 and 288 h, respectively.

Encapsulating or immobilising cells in gellan gum gives some protection to the bacteria as these bacteria can survive high caffeine concentrations when compared with the free cells. Immobilised cells can reduce abiotic and biotic stress, as well as providing protection for the cells from the toxic effects of hazardous compounds. The matrix minimises cell contact with the toxic compounds, thus increasing the survival of the cells (Hall et al., 1998). Moreover, it also increases cell growth and cell densities in various internal anaerobic and aerobic zones of the immobilising gel. In addition, immobilisation has been reported to enhance degradation activity by changing the metabolic characteristics of the living cells such as enzyme induction, cell growth, and yield (Ahmad et al., 2012; Chung et al., 2003). Based on this study, it is clear that immobilised cells of strain SIU shows a better caffeine degradation than free cells.

Figure 5 shows the caffeine degradation rates by immobilised and free bacterial cells incubated in caffeine concentrations ranging from 0.1 to 2.0 g/L. The rates of caffeine degradation for both immobilised and free cells were not initially similar at all caffeine concentrations. The immobilised cells showed a higher degradation rate than the free cells. The free cells stopped degrading caffeine completely at 1500 mg/L while the immobilised cells continued to degrade up to 2000 mg/L at a slow rate. This shows that the rate of caffeine degradation is increased by immobilisation due to the fact that immobilised cells degrade caffeine faster than the free-suspended cells. The result obtained shows that the optimum rate of caffeine degradation on immobilised cells occurs at a 500 mg/L caffeine concentration.

Reusability of immobilised beads

The reusability of immobilised cells in gellan gum was studied in several consecutive caffeine degradation processes. This is a paramount process in bioremediation to determine if there was deactivation of cells after being repeatedly used. Figure 6 shows the result for the reusability of immobilised *Leifsonia* sp. strain SIU to degrade 0.3 g/L caffeine. From the result obtained, the first cycle took 24 h to completely degrade caffeine and the caffeine-degrading ability decreased by 41% after the immobilised cells of strain SIU was used for at least 10 cycles. This result indicates that immobilised cells can be reused for at least nine consecutive complete degradation cycles without any decrease in caffeine-degrading activity. Several researchers have also carried out studies on the reusability of immobilised cells. Ahmad et al. (2012) reported that immobilised *Acinetobacter* sp. Strain AQ5NOL 1 can be used for 47 consecutive cycles, an immobilised consor-

tium of *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03 showed 20 times reusability (Liu et al., 2009), *Acinetobacter baumannii* SERDANG 1 showed 5 times reusability (Adinarayana et al., 2005), and Nedal et al. (2007) reported that immobilised phenol-degrading microorganism cells can be recycled for 3 consecutive cycles.

Based on this present study, the immobilised cells of *Leifsonia* sp. strain SIU showed a positive result on its continuous use in caffeine degradation. Therefore, the strain can be used efficiently to degrade caffeine for at least nine complete degradation cycles.

In conclusion, this work studied the degradation of caffeine by immobilised cells of *Leifsonia* sp. strain SIU immobilised in gellan gum microbeads. The strain was able to degrade up to 1500 mg/L and could be reused for at least nine cycles. These optimistic attributes will make it a suitable candidate for the bioremediation of caffeine and its derivatives produced by the coffee and tea industries.

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References

Adinarayana, K., Jyothi, B., and Ellaiah, P. (2005) Production of alkaline protease with immobilized cells of *B. subtilis* PE-11 in various matrices by entrapment technique. *AAPS Pharm. Sci. Tech*., **6**, 391–397.

Ahmad, S. A. (2008) Invertase production by *Bacillus macerans* immobilized on calcium alginate beads. *J. Appl. Sci. Res*., **4**, 1777–1781.

Ahmad, S. A., Shamaan, N. A., Arif, N. M., Koon, G. B., Shukor, M. Y. A. et al. (2012) Enhanced phenol degradation by immobilized *Acinetobacter* sp. strain AQ5NOL 1. *World J. Microbiol. Biotechnol*., **28**, 347–352.

Ahmad, S. A., Ibrahim, S., Shukor, M. Y., Johari, W. L. W., Rahman, N. A. A. et al. (2015) Biodegradation kinetics of caffeine by *Leifsonia* sp. strain sui. *J. Chem. Pharm. Sci*., **8**, 312–316.

Ashtaputre, A. A. and Shah, A. K. (1995) Studies on viscous, gel forming exopolysaccharide from *Sphingomonas paucimobilis* GS1. *Appl. Environ. Microbiol*., **61**, 1159–1162.

Baskaran, V. and Nemati, M. (2006) Anaerobic reduction of sulfate in immobilized bioreactor, using a microbial culture originated from an oil reservior. *Biochem. Eng. J*., **31**, 148–159.

Beshay, U. (2003) Production of alkaline protease by *Teredinobacter turnirae* cells immobilized in Ca-alginate beads. *Afr. J. Biotechnol*., **2**, 60–65.

Camelin, I., Lacroix, C., Paquin, C., Prevost, H., Cachon, R. et al. (1993) Effects of chelants on gellan gum rheological properties and setting temperature for immobilization of living Bifidobacteria. *Biotechnol. Prog*., **9**, 291–297.

Chibata, I., Tosa, T., and Takata, I. (1987) Immobilization of cells carrageenan. *Methods Enzymol*., **135**, 189–198.

Chung, T. P., Tseng, H. Y., and Juang, R. S. (2003) Mass transfer effect and intermediate detection for phenol degradation in immobilized *Pseudomonas putida* systems. *Proc. Biochem*., **38**, 1497–1507.

Doherty, E. S., Halling, P. J., and McNeil, B. (1995) The importance of bead size measurement in mass-transfer modelling with immobilized cells. *Appl. Microbiol. Biotechnol*., **43**, 440–444.

Duran, N. and Esposito, E. (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: A review. *Appl. Catal. B Environ*., **28**, 83–99.

Gibson, A. M., Morgan, R. M., and Nikitin, A. G. (2009) The effect of caffeine on the bacterial populations in a freshwater aquarium system. Student Summer Scholars. Paper 31.

Godjevargova, T., Mihova, S., and Gabrovska, K. (2004) Feed-bed biosorption of Cu^{2+} by polyacrylonitrile-immobilized dead cells of *Sacchaomycetes cerevisiae*. *World J. Microbiol. Biotechnol*., **20**, 273–279.

Gokulakrishnan, S., Chandraraj, K., and Gummadi, S. N. (2005) Microbial and enzymatic methods for the removal of caffeine. *Enzyme Microb. Technol*., **37**, 225–232.

Gosmann, B. and Rehm, H. J. (1986) Oxygen uptake of microorganisms entrapped in Ca-alginate. *Appl. Microbiol. Biotechnol*., **39**, 627–631.

Gummadi, S. N., Ganesh, K. B., and Devarai, S. (2009) Enhanced degradation of caffeine by immobilized cells of *Pseudomonas* sp. in agar-agar matrix using statistical approach. *Biochem. Eng. J*., **44**, 136–141.

Ha, J., Engler, C. R., and Wild, J. R. (2009) Biodegradation of coumaphos, chlorferon, and diethylthiophosphate using bacteria immobilized in Ca-alginate gel beads. *Bioresour. Technol*., **100**, 1138–1142.

Hall, B. M., Mcloughlin, A. J., Leung, K. T., Travors, J. T., and Lee, H. (1998) Transport and survival of alginate-encapsulated and free lux marked *Pseudomonas aeruginosa* UG2Lr cells in soil. *FEMS Microbiol. Ecol*., **26**, 51–61.

Ibrahim, S., Shukor, M. Y., Syed, M. A., Ab Rahman, N. A., Khalil, K. A. et al. (2014) Bacterial degradation of caffeine: A review. *Asian J. Plant Biol*., **2**, 24–33.

Ibrahim, S., Shukor, M. Y., Khalil, K. A., Halmi, M. I. E., Syed, M. A. et al. (2015a) Application of response surface methodology for optimising caffeine-degrading parameters by *Laifsonia* sp. strain SIU. *J. Environ. Biol*., **36**, 1215–1221.

Ibrahim, S., Shukor, M. Y., Syed, M. A., Johari, W. L. W., and Ahmad, S. A. (2015b) Characterisation and growth kinetics studies of caffeine-degrading bacterium *Leifsonia* sp. strain SIU. *Ann. Microbiol*., doi:10.1007/s13213-015-1108-z.

Kailasapathy, K. (2002) Microencapsulation of probiotic bacteria: technology and potential applications. *Curr. Issues Intest. Microbiol*., **3**, 39–48.

Kim, W. J., Kim, J. D., Kim, J., Oh, S. G., and Lee, Y. W. (2008) Elective caffeine removal from green tea using supercritical carbon dioxide extraction. *J. Food. Eng*., **89**, 303–309.

Lakshmi, V. and Das, N. (2013) Removal of caffeine from industrial wastewater using *Trichosporon asahii*. *J. Environ. Biol*., **34**, 701–708.

Liu, Y. J., Zhang, A. N., and Wang, X. C. (2009) Biodegradation of phenol by using free and immobilized cells of *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03. *Biochem. Eng. J*., **44**, 187–192.

Lorist, M. M. and Tops, M. (2003) Caffeine, fatigue, and cognition. *Brain Cogn*., **53**, 82–94.

Mazzafera, P. (1991) A cafeína do café. *Doc do Inst Agronômico Campinas*, **25**, 122.

Mazzafera, P. (2002) Degradation of caffeine by microorganisms and potential use of decaffeinated coffee husk and pulp in animal feeding. *Sci. Agric*., **59**, 815–821.

Miller, J. N. and Miller, J. C. (2002) Statistics and Chemometrics for Analytical Chemistry, 4th ed., Prentice Hall, Harlow, England, 288 pp.

Moslemy, P., Neufeld, R. J., and Guiot, S. R. (2002) Biodegradation of gasoline by gellan gum—Encapsulated bacterial cells. *Biotechnol. Bioeng*., **80**, 175–184.

Moslemy, P., Neufeld, R. J., Millette, D., and Guiot, S. R. (2003) Transport of gellan gum microbeads through sand: an experimental evaluation for encapsulated cell bioaugmentation. *J. Environ. Manage*., **69**, 249–259.

Nedal, M., Sobhi, B., and Islam, S. (2007) Effect of adsorption and bead size of immobilized biomass on the rate of biodegradation of phenol at high concentration levels. *Indian Eng. Chem. Res*., **46**, 6820–6824.

Norton, S. and Lacroix, C. (1990) Gellan gum gel as entrapped matrix for high temperature fermentation process-rheological study. *J. Chem. Technol. Biotechnol*., **4**, 351–356.

Pandey, A., Soccol, C. R., Nigam, P., Brand, D., Mohan, R. et al. (2000) Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochem. Eng. J*., **6**, 153–162.

Prabua, C. S. and Thatheyus, A. J. (2007) Biodegradation of acrylamide employing free and immobilized cells of *Pseudomonas aeruginosa*.

Int. Biodeterior. Biodegrad., **60**, 69–73.

Shukor, M. Y., Ahmad, S. A., Nadzir, M. M. M., Abdullah, M. P., Shamaan, N. A. et al. (2010) Molybdate reduction by *Pseudomonas* sp. strain DRY2. *J. Appl. Microbiol.*, **108**, 2050–2058.

Smith, A. (2002) Effects of caffeine on human behavior. *Food. Chem. Toxicol.*, **40**, 1243–1255.

Suzuki, T., Ashihara, H., and Waller, G. R. (1992) Purine and purine alkaloid metabolism in Camellia and Coffea plants. *Phytochemistry*, **31**, 2575–2584.

Vegesna, R. S. B. (2007) A Biotectechnological Approach for Decaffeination, 353 pp.

Waller, G. R., Kumari, D., Friedman, J., Friedman, N., and Chou, C. H. (1986) Caffeine autotoxicity in *Coffea arabica* L. *In* Advances in Allelopathy, ed. by Tang, C. S. and Putnam, A. R., John Wiley & Sons, New York.

Wang, J. L., Liu, P., and Qian, Y. (1997) Biodegradation of phthalic acid esters by immobilized microbial cells. *Environ. Int.*, **21**, 775–782.

Wang, Y., Tian, Y., Han, B., Zhao, H., Bi, J. et al. (2007) Biodegradation of phenol by free and immobilized *Acinetobacter* sp. strain PD12. *J. Environ. Sci.*, **19**, 222–225.

White, P. A. and Rasmussen, J. B. (1998) The genotoxic hazards of domestic wastes in surface waters. *Mutat. Res.*, **410**, 223–236.

Wijhe, M. V. (2002) The history of caffeine as used in anaesthesia. *Int. Congr. Ser.*, **1242**, 101–103.