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ORIGINAL ARTICLE

Characterisation and growth kinetics studies of caffeine-degrading bacterium *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 effects. We isolated a new bacterium from agriculture soil. The bacterium was tested for its ability to utilise caffeine as the sole carbon and nitrogen source. The isolate was Gram-negative and was identified as *Leifsonia* sp. strain SIU based on 16S rRNA gene sequencing. It showed 97.16 % of 0.3 g/L caffeine degradation in 48 h when caffeine was used as a sole carbon and nitrogen source. The bacterial growth and degradation at 0.3 g/L caffeine concentration occurred optimally, using 5 g/L sucrose, 0.4 g/L ammonium chloride, at a temperature between 25 and 30 °C and pH of 6.0–7.0. The Luong model best describes the kinetics of the strain growth. The values for the maximum specific growth rate (μ_{max}), the Monod half saturation constant (K_S), the maximum substrate inhibitory concentration and n are 0.049 h^{-1}, 0.0021 mg/L, 25.0 g/L and 1.562, respectively. These bacterial features make it an ultimate means for caffeine bioremediation. This is the first report of caffeine degradation by *Leifsonia* sp. strain SIU.

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Introduction

Caffeine (1,3,7-trimethylxanthine) is a member of compounds known as purine alkaloids. It is found in more than 60 plants species, including coffee seeds, tea leaves, cocoa, kola nut, guarana, and yerba mate (Ashihara and Crozier 2001). Because of its stimulatory effects, caffeine is used as a key component in beverages, especially tea and coffee. Excessive caffeine consumption can lead to a number of health complications such as increased cardiac output, gastrointestinal problems, swings in blood sugar, headache, fatigue, apathy, adrenal stimulation, irregular muscular activity, cardiac arrhythmias, and osteoporosis, as well as deleterious effect on cardiac patients (Smith 2002; Lorist and Tops 2003). Excessive caffeine consumption can cause mutation. Caffeine is teratogenic, causes inhibition of DNA repair, inhibition of cyclic AMP phoshodiesterase activity, and inhibits seed germination (Friedman and Waller 1983a, b). Caffeine is a major cause of cancer, heart disease, and complications in aging and pregnant women, as it increases the spontaneous risk of abortion and affects foetal growth, thus causing malfunction in the foetus (Srisuphan and Bracken 1986; Fenster et al. 1991; Infante et al. 1993; Dlugosz et al. 1996; Green and Suls 1996). Apart from the health effects of caffeine, degradation of caffeine is important from an environmental point of view. Wastes such as tea, husk, and coffee pulp generated from tea and coffee industries are major toxic compounds. Even though these wastes are rich in proteins and carbohydrates, due to the presence of anti-nutritional factors such as caffeine, tannins, polyphenols and other harmful substances, they have limited use as animal feeds (Mazzafera 2002; Gummadi et al.

2012). Moreover, channelling of these industrial wastes into nearby water bodies like rivers and lakes would affect the marine environment (White and Rasmussen 1998; Gibson et al. 2009). Caffeine present in the soil also affects soil fertility, as the compound inhibits growth of seedlings and seed germination (Batish et al. 2008; Lakshmi and Nilanjana 2013a). Therefore, there is a strong need for caffeine degradation from products and waste streams by routes other than conventional extraction techniques (Gokulakrishnan et al. 2005; Mohanty 2013).

Conventional decaffeination methods such as supercritical fluid and solvent extraction are non-specific, expensive, and involve the use of poisonous solvents that are toxic to the environment. All these methods lead to the loss of aroma and flavour of the compounds, and result in food products deprived of value. Thus, in developing biodecaffeination methods, microorganisms and enzymes acquired from microbial systems should be studied, as these microorganisms and enzymes are more specific, more eco-friendly, easier to use, and cheaper (Asano et al. 1994). Therefore, in the recent past, microbial degradation of caffeine has become the focus of research, owing its major advantages over conventional techniques of decaffeination (Gokulakrishnan et al. 2005; Gummadi et al. 2012). Caffeine degradation has been well studied in bacterial strains, primarily in the genus *Pseudomonas*, and others such as *Serratia*, *Rhodococcus* and *Klebsiella* species (Gummadi et al. 2012). Unlike higher organisms, bacteria utilise caffeine as the sole source of nitrogen, carbon and energy for growth (Yu et al. 2008; Yu et al. 2009; Mohanty et al. 2012). Over the past few decades, shake flask experiments to lab-scale batch processes and solid-state fermentation processes have been developed, making biodecaffeination a plausible method in wastewater treatment (Mazzafera et al. 1996; Babu et al. 2005). Furthermore, several caffeine-degrading bacteria have been isolated, which can be further processed for successful decaffeination of food products (Mohapatra et al. 2006). The development of a biotechnological process of decaffeination still remains a distant certainty due to various issues, such as the requirement of co-factors, extreme liability of enzymes, and complex problems like enzyme inhibition activities involved in the decaffeination pathway. Due to these process factors, microbial caffeine decaffeination has not been developed for commercial purposes. A bacterium such as *Leifsonia* sp. has many applications in treatment technology. It was found to degrade dihydropyrimidines and hydantoins, which were identified for the degradation of diketopiperazines (DKPs) (Perzborn et al. 2013). It is a coryneform bacterium and one of the most economically important pathogens of sugarcane worldwide, causing ratoon stunting disease, with losses in infected plants ranging from less than 5 % to more than 30 % (Bailey and Bechet 1997; Brumbley et al. 2006). Some essential factors for the biotechnological development of a process for microbial caffeine degradation are: (1) temperature; (2) pH; (3) rate of caffeine degradation; (4) nitrogen source; (5) carbon source; and (6) initial caffeine concentration. Table 1 shows caffeine degradation rates by various microorganisms.

In this study, we isolated a new bacterium, namely *Leifsonia* sp. strain SIU, from Malaysian agricultural soil. As it is highly influenced by sugars, nitrogen source and pH, this bacterial strain has the capability to degrade caffeine.

Materials and methods

Chemicals and media

Caffeine, anhydrous > 99 %, was purchased from Sigma, Aldrich USA. Other chemicals that were of analytical grade were obtained from recognised chemicals suppliers: Fisher (Malaysia) and Merck (Darmstadt, Germany). The medium used contained the following (in 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 $NaMoO_4{\cdot}2H_2O$, 0.4 NH_4Cl. The caffeine medium consisted of these salts supplemented with 0.3 g/L of caffeine. Carbon sources, if added to the medium, were sterilised separately and then mixed into the medium under aseptic conditions. For solid medium, agar (25 g/L) was added to the caffeine medium. The isolates were maintained and subcultured every 2 weeks in the caffeine agar medium.

Isolation of caffeine-degrading bacteria

Soil samples used in this work were collected from various areas across Malaysia at 30 cm depth from soil surface under aseptic conditions. Serial dilutions of the soil sample (1/10, $1/10^2$, $1/10^3$, $1/10^4$, and $1/10^5$) were prepared in sterile caffeine minimum salt medium and plated on caffeine agar medium plates, which were then incubated and at 30 °C for 48 h. The isolates that utilised caffeine as a sole carbon and nitrogen source were picked and sub-cultured every 2 weeks. Seed cultures were prepared in 100 mL Erlenmeyer flasks containing 25 mL of sterilised nutrient broth medium with 1 mL of bacterial suspension, and incubated on a rotary shaker (150 rpm) at room temperature for 24 h until the OD_{600} reached 1.3–1.4. These were used as standard inocula (1 mL/25 mL medium) unless otherwise stated.

Flask culture experiments

A single colony of the strain from caffeine agar plates was transferred to 5 mL sterile caffeine medium. Tubes with cotton plugs were aerated on a rotary shaker at 150 rpm and incubated for 24 h at room temperature. About 4 % (v/v) of the culture was transferred to 50 mL of the caffeine medium in 250 mL

Table 1 Caffeine degradation rates by various microorganisms

Microorganisms	Initial caffeine concentration (g/L)	Carbon source (g/L)	Caffeine degradation (%)	Temp. (°C)	pH	Reference
Klebsiella and *Rhodococcus*	0.5	Glucose (1)	100 in 10 h	–	–	Madyastha and Sridhar 1998
Serratia marcescens	0.6	–	100 in 72 h	–	–	Mazzafera et al. 1996
Pseudomonas putida	5	Sucrose (30.1)	67.2 in 48 h	–	–	Woolfolk 1975
Pseudomonas sp GSC 1182	5	Sucrose (5)	80 in 48 h	30	6	Gokulakrishnan et al. 2007
Pseudomonas alcaligens CFR 1708	1	–	–	30	7.0–8.0	Babu et al. 2005
Pseudomonas sp. immobilised in agar-agar matrix	10	–	–	–	–	Gummadi et al. 2009
Brevibacterium sp.	1–8	Glucose and sucrose	–	–	–	Nayak et al. 2012
Pseudomonas stutzeri Gr 21 ZF	1.2	Sucrose (5)	80 in 48 h	30	7	El-Mched et al. 2013

Erlenmeyer flasks and incubated on a rotary shaker at 150 rpm for 48 h at 30 °C. Samples were collected after 48 h and cell growth and caffeine degradation were measured.

Caffeine-degrading bacterium identification

The bacterium was genetically identified through 16S rRNA gene sequencing and Gram staining. Gram staining was carried out according to the standard procedure, as reported by Cappucino and Sherman (2005). Genomic DNA was prepared through an alkaline lysis method using the thermo-scientific GeneJET Genomic DNA purification kit. PCR amplification was carried out using a Biometra T Gradient PCR Thermal Cycler (Montreal Biotech Inc., Kirkland, QC). The PCR mixture contained forward primer (5′ tac gga tac ctt gtt acg act t 3′) and reverse primer (5′ aga gtt tga tcc tgg ctc ag 3′), both of which were synthesised from First Base for the amplification of the 16S rRNA region (Devereux and Wilkinson 2004). The DNA amplification was done using a thermal cycler (MJ Research Inc. USA). About 25 μL of the PCR reaction mixture was added to a 0.5 mL thin-walled PCR tube. Afterwards, the samples were placed in the thermal cycler for the process of amplification. After initial denaturation for 1 min at 94 °C, the DNA was amplified for 30 cycles, annealing for 2 min at 58 °C and extending for 2 min at 72 °C, followed by the final extension for 10 min at 72 °C (Sambrook et al. 1989). The resultant 1484 base pairs were compared to similar sequences in the GenBank database using the NCBI Blast server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rRNA ribosomal gene sequence was submitted to GenBank under the accession number KJ191763.

Phylogenetic analysis

Twenty closely interrelated 16S rRNA gene sequences were recovered from GenBank and were compared with *Leifsonia* sp. and aligned using Clustal W (Thompson et al. 1994). Construction of the phylogenetic tree was carried out using PHYLIP Version 3.573 (J.Q. Felsenstein, PHYLIP-phylogeny inference package, version 3.573, Department of Genetics, University of Washinton, Seattle, WA., http://evolution.genetics.washinton.edu/phylip.html), with *Serratia marcescens* as an outgroup in the cladogram. The model used in the nucleotide substitution was from Jukes and Cantor (1969). A phylogenetic tree (Fig. 1) was constructed using the neighbour-joining method of Saitou and Nei (1987). With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1000 bootstraps (Felsenstein 1985), using the SEQBOOT program in the PHYLIP package. Majority rule (50 %) consensus tree methods, called MI methods, were used with the CONSENSE program, and the tree was viewed using Tree View (Page 1996).

Analytical method

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 the 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) (Gokulakrishnan et al. 2007). Cell density in the medium was monitored by measuring the optical density at 600 nm.

Growth kinetics modelling experiment

A batch experiment was carried out using shake flask studies at optimal conditions for caffeine degradation by strain SIU. The flask was incubated for 48 h at room temperature and 150 rpm. About 4 % (v/v) of the seed culture was transferred to 100 mL Erlenmeyer flasks with 25 mL of caffeine liquid media containing various initial caffeine concentrations ranging from 300 to

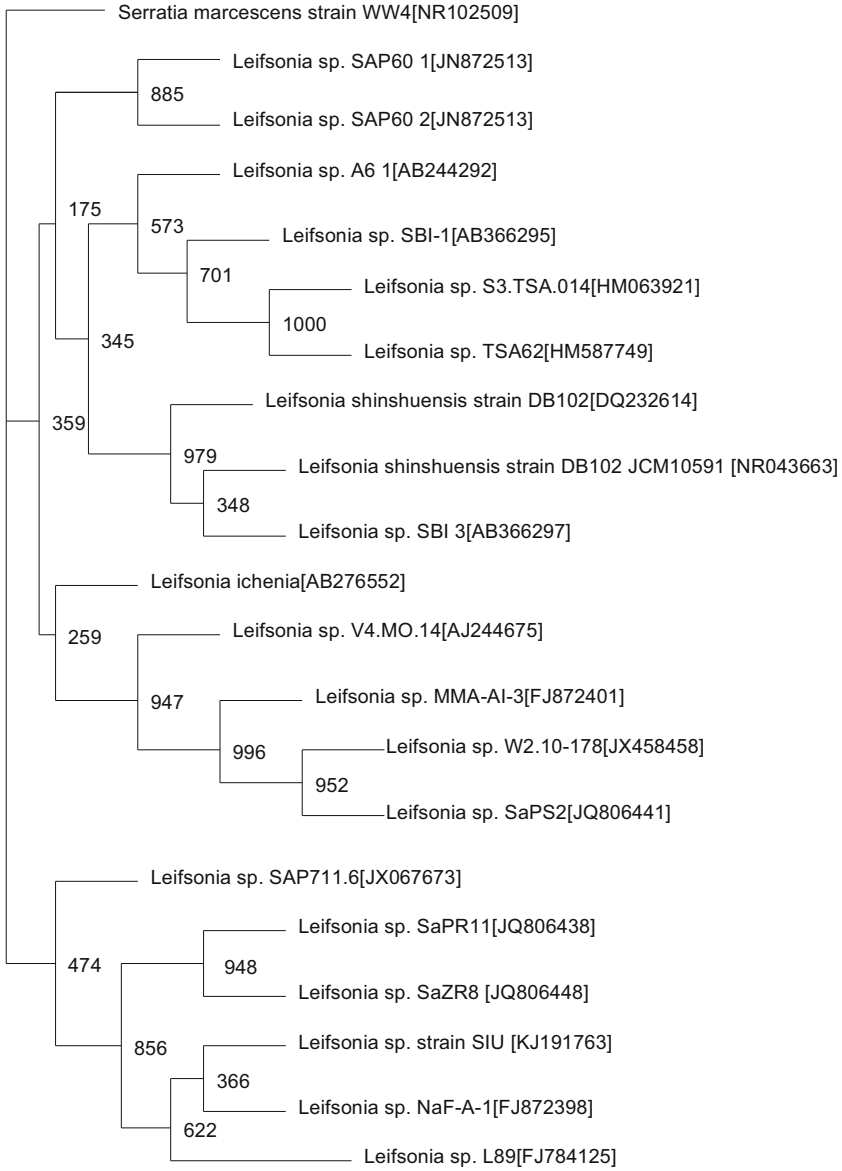

Fig. 1 Phylogram (neighbour-joining method) indicating the 16S rRNA genetic relationship between strain SIU and 20 other related references microorganisms from the GenBank database. *S. marcescens* is used as an outgroup. Species names of bacteria are followed by 16S rRNA accession numbers. The internal labels at the branching points are the bootstrap value. Scale bar represents 100 nucleotide substitution

15,000 mg/L, and incubated on a rotary shaker at 150 rpm and at room temperature. Samples were collected at different time intervals and measured for cell growth (Gokulakrishnan and Gummadi 2006; Agarwal et al. 2009). In this study, growth kinetic models as listed in Table 2 were used to describe the growth kinetics of the caffeine-degrading bacterium. All of the growth kinetic models were fitted to the experimental data using MATLAB R2012a (Singh et al. 2008).

The rate of bacterial growth can be represented as cell production rate. The formulas for various kinetics models are shown in Table 2 where S, S_m, μ, μ_{max}, K_s, K_i, and n are specific substrate concentration (mg/L), the above critical substrate concentration above which cell growth of caffeine completely stops (mg/L), cell growth rate (h^{-1}), maximum cell growth rate (h^{-1}), saturation constant or half velocity constant (mg/L), inhibition constant (mg/L), and the exponent representing the impact of the substrate to μ_{max}, respectively. For each initial concentration of caffeine, specific growth rate was calculated based on the linear portion of the growth against time in an exponential phase. The specific growth rate

Table 2 Various kinetic models for the effect of caffeine substrate on cell growth

Model	μ (Growth rate)	References
Haldane	$\mu_{max}\frac{S}{S+K_s+\left(\frac{S^2}{K_i}\right)}$	(Haldane 1930)
Luong	$\mu_{max}\frac{S}{K_s+S}\left(1-\frac{S}{S_m}\right)^n$	(Luong 1987)
Aiba-Edwards	$\mu_{max}\frac{S}{K_s+S}exp^{(-S/K_i)}$	(Aiba et al. 1968)

(μ) in exponential phase was calculated by the following equation:

$$\mu = \frac{\ln\left(X_2 / X_1\right)}{t_2 - t_1} \quad (1)$$

where X_1 and X_2 are the cell dry weight obtained at time t_1 and t_2, respectively. All experiments were conducted in triplicate under identical conditions, and all results had a mean standard deviation (Gokulakrishnan and Gummadi 2006).

Characterisation of caffeine-degrading bacterium

Optimisation of growth and caffeine-degradation parameters, i.e., concentration of caffeine, different carbon and nitrogen sources, pH, and temperature, was carried out for strain SIU. Various carbon sources, such as glucose, sucrose, fructose, mannitol, maltose, dextrin, starch, lactose, galactose, and cellulose, were added to the caffeine medium at 5 g/L, and the bacterial growth, carbon source utilisation and caffeine degradation were measured. Concentrations of sucrose ranging from 0.1 to 0.8 % (w/v) were subsequently tested with respect to growth and degradation by strain SIU.

The effect of temperature on caffeine degradation was determined by measuring caffeine utilisation and cell growth after a 48 h incubation period at different incubation temperatures (10, 15, 20, 25, 27, 30, 35, 37, and 40 °C). In a separate experiment, the effect of pH was studied by varying the initial pH of the medium between 4 and 9. Various concentrations of caffeine, ranging from 0.05 to 2.0 g/L, were also tested.

In order to study the effect of an additional nitrogen source on caffeine degradation, caffeine medium with 5 g/L sucrose was supplemented, and nitrogen sources such as NH_4NO_3, NH_4Cl, NH_4CO_3, glutamate, aspartate, NH_4SO_4 and glycine were added to the caffeine media separately at a concentration of 0.4 g/L. Media without any nitrogen source addition served as a control for the experiment. All experiments were performed in triplicate under identical conditions and the data presented are means of triplicate experiments.

Statistical analysis

The average deviations (three replicates) of the samples were statistically analysed using Design Expert 6.0, using first-order factorial design.

Results and discussion

Identification of a caffeine-degrading bacterium

We have isolated a new bacterium, strain SIU, with the ability to degrade caffeine from agriculture soil in Malaysia. The bacterium is Gram-negative, spore-forming, rod-shaped, and is capable of degrading caffeine. The bacterium was identified through phylogenetic analysis of its 16S rRNA ribosomal gene sequence.

A homology search with the blasting tool revealed that the sequence of 16S rRNA gene from SIU was 99 % identical with the corresponding sequences of *Leifsonia* sp. A low bootstrap value (<70 %) of 36.6 % was obtained when the strain SIU was genetically linked to *Leifsonia* sp. (Fig. 1). This bacterium is a novel, caffeine-degrading, Gram-negative bacterium. At this juncture, we have tentatively assigned this bacterium as *Leifsonia* sp. strain SIU.

Modelling kinetics

Caffeine growth kinetics were determined by measuring the cell growth rate at different times for 50 h at different initial concentrations of caffeine. Figure 2 shows the resulting bacterial growth curve of *Leifsonia* sp. strain SIU at different caffeine concentrations. The cell growth increased, reaching an optimal concentration at 300 mg/L, and then it started to decrease with an increase in caffeine concentration. From the result obtained in Fig. 2, it can be concluded that high caffeine substrate has an effect on the growth of *Leifsonia* sp. strain SIU. The optimal growth of the bacteria was found to be 300 mg/L with an OD_{600} of 2.306; then it started to decline with an increase in substrate until the substrate completely inhibited the growth of the bacteria. It was found that a substrate concentration of 15,000 mg/L completely inhibits the growth of the bacteria, with an OD_{600} of 0.093.

The relationship between the specific growth rate (μ) of a population of microorganisms and the substrate (caffeine) concentration (S) is an important factor in the area of biotechnology. This association is characterised by a set of empirically derived rate laws called theoretical models. These models are nothing more than numerical expressions created to describe the behaviour of a given system. Based on the growth curves of *Leifsonia* sp., the specific growth rate (μ) for each initial caffeine concentration (S) was calculated. The gradient of line during the exponential phase provided the specific

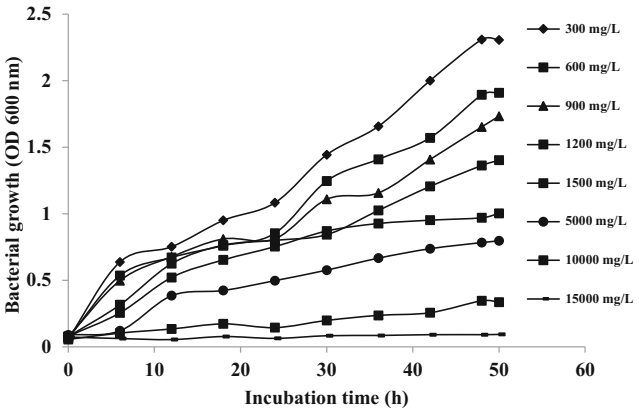

Fig. 2 Effect of different caffeine concentrations on strain SIU growth

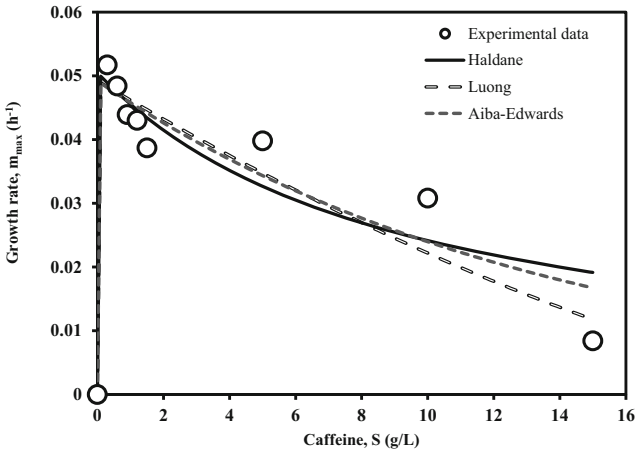

Fig. 3 Comparison of caffeine growth kinetic experimental values with three different kinetic models

growth rate (Fig. 3). The plot shows a definite increase in cell growth rate with increase in caffeine concentration until 300 mg/L, beyond which there was a decrease in cell growth rate as caffeine concentration increased, signifying caffeine inhibition kinetics.

Data from the experimental values in batch studies were fitted to kinetic models (Fig. 3). Table 3 presents the results of kinetic models with correlation coefficient R^2. The Luong model describes the kinetics of the strain's growth better than the Haldane and Aiba-Edwards models. The values for the maximum specific growth rate (μ_{max}), the Monod half saturation constant (K_S), the maximum substrate inhibitory concentration (S_m) and n are 0.049 h^{-1}, 0.0021 mg/L, 25.0 g/L and 1.562, respectively. The value of μ_{max} estimated by Luong model (0.04922 h^{-1}) was closer to the experimental value of 0.0517 h^{-1} obtained at 300 mg/L. The value of S_m predicted by the Luong model (25.0 g/L) indicates that at and beyond this concentration, no growth on caffeine will be observed. The constant n was estimated to be greater than 1, indicating a non-linear correlation between specific growth and the initial substrate concentration. These estimations of parameters suggest that the Luong model best describes the inhibition kinetics of cell growth.

Table 3 Parameter estimations for different substrate-inhibition models

Model	Parameter	Unit	Value	R^2
Haldane	μ_{max}	h^{-1}	0.0505	0.9035
	K_S	mg/L	0.008631	
	K_I	mg/L	9161	
Luong	μ_{max}	h^{-1}	0.04922	0.9329
	K_S	mg/L	0.002094	
	S_m	mg/L	25,030	
	N		1.562	
Aiba-Edwards	μ_{max}	h^{-1}	0.04921	0.9226
	K_S	mg/L	0.001143	
	K_I	mg/L	13,900	

Effect of growth parameters

The effect of various growth parameters such as temperature, pH, caffeine concentration, carbon and nitrogen source on caffeine degradation was studied. The bacterium was grown in a minimal salt medium containing 0.3 g/L of caffeine in 250 mL conical flasks and incubated at room temperature on a rotary shaker (150 rpm) for 48 h. All experiments were conducted in triplicate under the same conditions and the mean was taken. The same medium with varying caffeine concentrations was used to study the effect of initial caffeine concentration on caffeine degradation.

Effect of various carbon sources

Ten carbon sources, namely glucose, sucrose, fructose, mannitol, maltose, dextrin, starch, lactose, galactose, and cellulose, were used at an initial percentage of 0.5 % (w/v). Roussos et al. (1994) and Hakil et al. (1999) reported that different carbon sources have different effects on caffeine degradation. The carbon sources were sterilised separately and added to sterile caffeine media. The results (Fig. 4) show that sucrose (97.16 %) was the best carbon source for bacterial growth, followed by galactose (96.48 %) and fructose (74.77 %) after 48 h of incubation. All these sources supported caffeine degradation, while others only supported growth. Cellulose has an inhibitory effect on caffeine degradation (4.56 %). All the remaining carbon sources that were tested, as shown in Fig. 4, showed an inhibitory effect on caffeine degradation by strain SIU. Madyastha and Sridhar (1998) reported that the best carbon source supporting caffeine degradation for *Klebsiella* and *Rhodococcus* is glucose, while Gokulakrishnan and Gummadi (2006) revealed that caffeine degradation by *Pseudomonas* sp. GSC 1182 was inhibited in the presence of glucose and was enhanced by the presence of sucrose and lactose. This result was in compliance with the result obtained by Hakil et al. (1999) and Lakshmi and Nilanjana

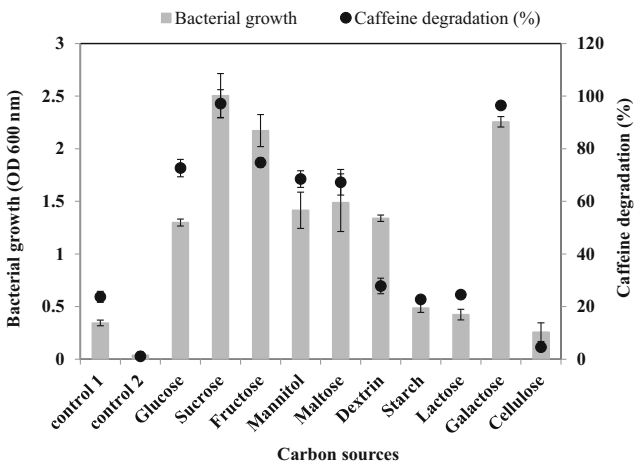

Fig. 4 Effect of Carbon source on caffeine degradation by *Leifsonia* sp. strain SIU. *Error bars* represent mean ± standard deviation (n = 3)

(2013b), who reported that caffeine degradation by *Penicillum* sp., *Aspergillus*, and *Trichosporon asahii* can be increased by the addition of sucrose. On the other hand, it has been reported that certain bacteria such as *Serratia marcescens* and *Pseudomonas putida* have the ability to degrade caffeine in the absence of glucose or sucrose or any other carbon sources in the medium (Woolfolk 1975; Mazzafera et al. 1996). Control 1 is the medium without the addition of any carbon source, while control 2 is without the addition of bacteria, and there was no degradation recorded in the negative control 2 at the end of the experiment.

Effect of sucrose concentration

It is believed that a high concentration of carbon source is toxic to microorganisms. Based on the results obtained above, sucrose was found to be the best carbon source for the growth of and degradation by *Leifsonia* sp. strain SIU. As such, the concentration of sucrose was varied from 0.1 to 0.8 % in order to determine at what concentration the carbon source inhibited bacterial growth and degradation. The optimal concentration of sucrose for maximal growth of *Leifsonia* sp. strain SIU was found to be 0.5 % (w/v) (Fig. 5). The growth and degradation was slightly low at 0.4 and 0.7 % sucrose, but growth was still higher than for other carbon sources. At concentrations higher than 0.5 % (w/v), the growth was dramatically lowered, probably due to osmotic pressure (Alden et al. 2001).

Effect of temperature

Temperature is one of the major factors to be considered in caffeine degradation, as it has an effect on most biochemical reactions. In this study, the effects of temperature on the caffeine degradation and growth of the bacterium were studied at temperatures ranging from 10 to 40 °C. Figure 6 shows that the bacterium can grow at a temperature range of 25–30 °C, with an optimal temperature of 27 °C (95.93 %), after which there

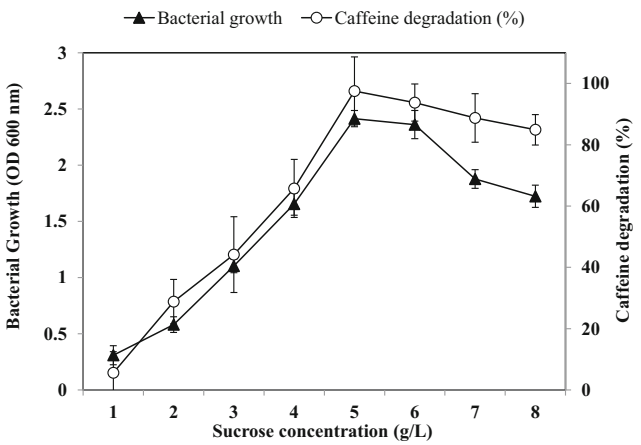

Fig. 5 Effect of sucrose on bacterial growth and degradation. *Error bars* represent mean ± standard deviation (n = 3)

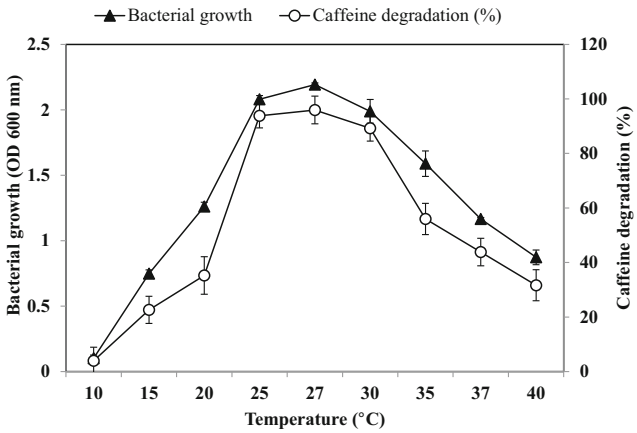

Fig. 6 The effect of temperature on caffeine degradation by *Leifsonia* sp. strain SIU. *Error bars* represent mean ± standard deviation (n = 3)

was a decline. At 10 °C the bacterial growth and degradation was very low, showing that the strain cannot withstand low temperature. The degradative capacity of *Leifsonia* sp. strain SIU was fairly good at 20, 35, and 37 °C. Caffeine degradation was less than 35 % at 15 and 40 °C. Lakshmi and Das (2010) reported that *Trichosporon asahii* degrades 73 % at 28 °C, while in a mixed culture of *Klebsiella* and *Rhodococcus* sp., the optimal temperature was 40 °C (Madyastha et al. 1999). Caffeine degradation in *Pseudomonas* sp. GSC 1182 shows 80 % degradation at 30 °C within 48 h (Gokulakrishnan et al. 2007), and in *Pseudomonas alcaligenes* it was 35 °C (Mohapatra et al. 2006).

Effect of initial pH

pH is an important physical parameter that affects the growth and metabolism of all microorganisms. pH also plays a significant role in the activity of various enzymes required for caffeine degradation and bacterial growth. Changes in pH have a major effect on the bacterial enzymes such as caffeine demethylase, an enzyme that is involved in caffeine degradation. As such, in order to study the effect of pH on caffeine degradation by *Leifsonia* sp. strain SIU, the initial pH of the caffeine medium was varied from pH 3 to pH 9. Figure 7 shows that the bacterium can grow at a pH range between pH 6 and pH 7, with an optimal pH of 6.5 (97.11 %), after which there is a decline in the pH. At extreme pH such as 3, 4, and 9, the caffeine degradation values were 2.69, 3.99 and 4.79 %, respectively, while at pH 5, pH 5.5, and pH 8, the caffeine degradation values were 22.46, 51.28, and 55.53 %, respectively. These results mean that the strain is a neutrophilic bacterium. Previous study shows that high level of caffeine degradation in *Trichosporon asahii* was observed at pH 6.5 (Lakshmi and Das 2010). This finding is contrary to the results obtained by El-Mched et al. (2013), who reported that maximal caffeine degradation for *Pseudomonas stutzeri* Gr 21 ZF was at pH 7.0, while maximal caffeine degradation for mixed

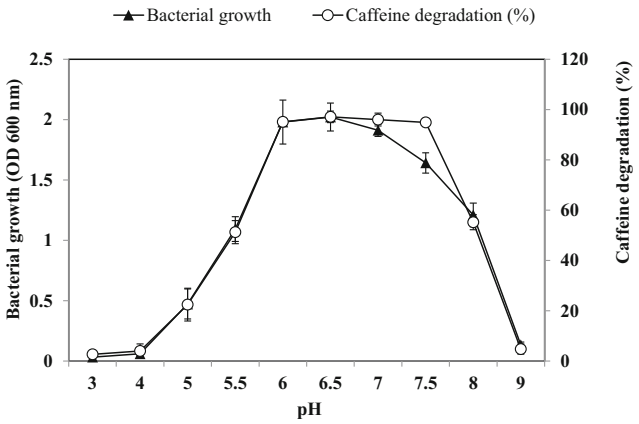

Fig. 7 The effect of pH on caffeine degradation by *Leifsonia* sp. strain SIU. *Error bars* represent mean ± standard deviation ($n = 3$)

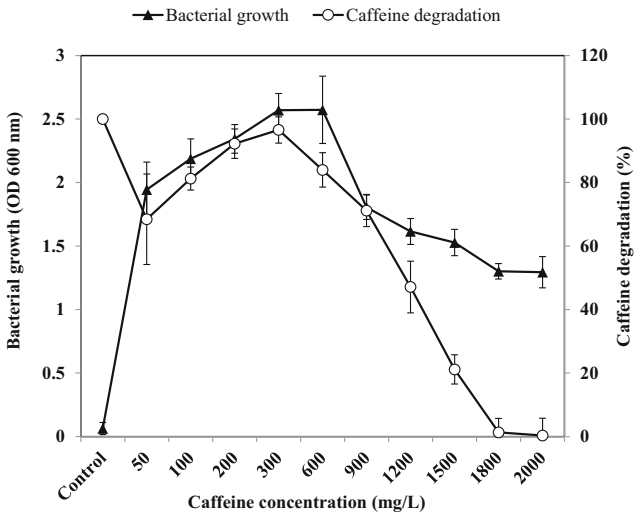

Fig. 8 The effect of caffeine concentration on caffeine degradation by *Leifsonia* sp. strain SIU. *Error bars* represent mean ± standard deviation ($n = 3$)

culture of *Klebsiella* and *Rhodococcus* was found to be at pH 7.5 (Madyastha et al. 1999). Babu et al. (2005) reported that *Pseudomonas alcaligenes* CFR 1708 showed the highest caffeine degradation capacity at a pH range of 7.0–8.0. Lakshmi and Das (2010) also reported that the optimum for *Trichosporon asahii* was in the acidic pH range (5.5–6.5), while Gokulakrishnan et al. (2007) in a separate research reported that the optimal degradation pH for caffeine is 6.0 for *Pseudomonas* sp. GSC 1182 and at this pH, the time required for complete caffeine degradation was reduced from 48 to 24 h.

Effects of caffeine concentration

Caffeine is known to be toxic to microorganisms at high concentrations, and efficient biodecaffeination demands the selection of a threshold level of caffeine that the microorganisms can tolerate and degrade efficiently. Nonetheless, caffeine at high concentrations is inhibitory to the growth of microorganisms (Nathanson 1984; Frischknecht et al. 1986; Asano et al. 1993). Thus, optimisation of caffeine concentration is paramount in order to obtain maximal bacterial growth and degradation. In this study, the *Leifsonia* sp. strain SIU was incubated with caffeine in the concentration range of 0.05-2.00 g/L (Fig. 8). The optimum caffeine-degrading capacity was found to be at a concentration of 0.3 g/L, with an optimum degradation of 96.55 % after 48 h of incubation. At high concentrations, caffeine was found to be inhibitory to microorganisms, with 47.10, 21.14, 1.28, and 0.32 % of caffeine degradation at caffeine concentrations of 1.2, 1.5, 1.8, and 2.0 g/L, respectively.

As reported by Woolfolk (1975), in the study of caffeine degradation of *Pseudomonas putida* at a concentration of 5 g/L, the culture required many years of maintenance on caffeine medium (1 g/L) and the degradation was extremely sluggish, requiring about 1 week. Numerous bacterial strains belonging to *Rhodococcus*, *Klebsiella*, *Acinetobacter, Brevibacterium* sp., *Serratia*, *Pseudomonas*, etc. have been reported in the literature on caffeine degradation (Gummadi et al. 2009; Mazzafera et al. 1996: Nayak et al. 2012). All these microorganisms are known to degrade caffeine, but they are extremely sluggish. In contrast, our new isolated bacterium strain SIU was capable of degrading 0.3 g/L caffeine within 48 h of incubation. As such, this isolate showed the best growth and degradation in medium containing caffeine as the sole source of carbon and nitrogen within 30–48 h of incubation. The Control was medium without the addition of any caffeine source, and no bacterial growth was recorded for the control at the end of the experiment.

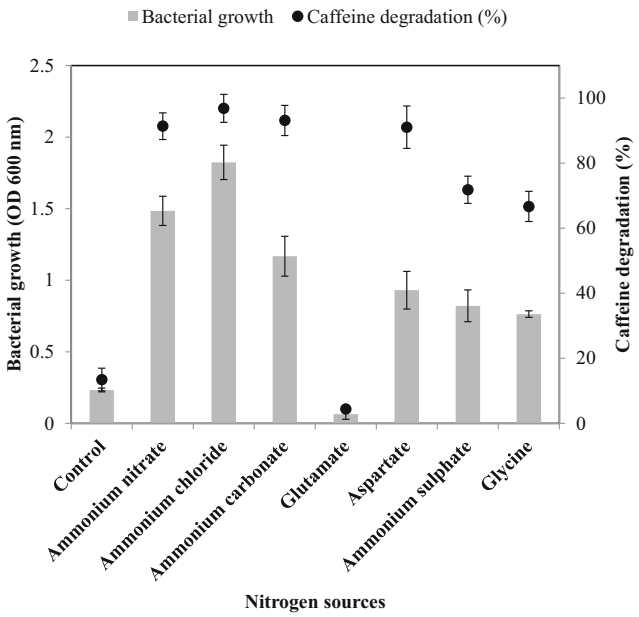

Fig. 9 Effect of nitrogen source on caffeine degradation by *Leifsonia* sp. strain SIU. *Error bars* represent mean ± standard deviation ($n = 3$)

Effects of nitrogen source

Nitrogen source is one of the most important elements in bacterial composition, apart from carbon source. In this study, seven organic and inorganic nitrogen sources, namely ammonium nitrate, ammonium chloride, ammonium carbonate, glutamate, aspartate, ammonium sulphate and glycine, were tested as nitrogen sources. The nitrogen content was taken into consideration, as the total carbon in 0.04 % (w/v) of each nitrogen source was different. The results (Fig. 9) show that ammonium chloride (96.85 %) was the best nitrogen source for bacterial growth and degradation, followed by ammonium nitrate (91.37 %) and ammonium carbonate (93.12 %) after 48 h of incubation. All these sources supported caffeine degradation, while others only supported growth. The addition of external nitrogen sources inhibited caffeine degradation. The inhibitory effect was stronger for glutamate than glycine. The presence of glutamate had a strong inhibitory effect on caffeine degradation (4.43 %). It was found that the presence of the above-mentioned additional nitrogen sources in the caffeine salt medium inhibited caffeine degradation. These results were in compliance with the results obtained by Lakshmi and Nilanjana (2013b). It has been reported that an external supply of nitrogen source improves the degradation of caffeine in *Pseudomonas* sp. Tryptone at 0.5 % (w/v) was found to be the most effective nitrogen source (Asano et al. 1993), but this is in contrast to the results obtained by Gokulakrishnan et al. (2007), who reported that the addition of external nitrogen source (either organic and inorganic) inhibited the caffeine degradation, and the inhibitory effect was stronger for urea and ammonium sulphate in *Pseudomonas* sp. GSC 1182. Roussos et al. (1994) reported that the addition of nitrogen sources in the medium inhibited caffeine degradation. The control was medium without the addition of any nitrogen source, and degradation was found to be 13.46 % at the end of the experiment.

Conclusion

We report the isolation of the first caffeine-degrading bacterium from the *Leifsonia* genus isolated from agricultural soil. The isolated strain SIU is a new, efficient caffeine degrader, which may be useful in the development of an environmentally friendly biodecaffeination process. We report for the first time that the Luong kinetic model can best fit the experimental data from this bacterium. Degradation efficiency was found to be affected by some parameters such as pH, temperature, initial caffeine concentration, and additional carbon and nitrogen sources. The present study helped us to understand that caffeine degradation may be enhanced by additional carbon sources such as sucrose. The present information regarding the effective bacterial degradation of caffeine can be useful to the real state where degradation is essential in caffeine contaminated areas.

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