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Full Length Research Paper

Isolation and characterization of high caffeine-tolerant bacterium strains from the soil of tea garden

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Microbial biodegradation is an important and promising decaffeination approach because of its low cost and high security. The efficiency of this approach mainly depends on the characteristics of the strain. Twenty bacterium strains were isolated from soil of tea garden and exhibited high caffeine-tolerance, and these strains could grow on the medium supplemented with 20 g L^{-1} caffeine as the sole source for nitrogen and carbon. According to the physiological-biochemical characteristics and 16S rRNA gene sequence blast, two of them (CT25 and CT75) were identified as *Pseudomonas putida*. The results also showed that caffeine was not the preferential nutrition source for growth of strain CT25, and high level caffeine inhibited the bacterium amplification although caffeine could be metabolized by this strain. The CT25 grew well in agitated liquid medium when the incubation temperature was around 30°C. High initial concentration of strain inoculums would improve the efficiency of caffeine degradation.

Key words: Caffeine-tolerant bacterium, identification, 16S rRNA gene, caffeine biodegradation, growth condition.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine), a member of purine alkaloids, was isolated first from tea and coffee in the early 1820s and then reported in the beans, leaves and fruits of more than 60 plants, such as coffee, tea, mate, guarana, cola and cocoa (Heckman et al., 2010). Medicinally, caffeine is used as a cardiac, cerebral and respiratory stimulant (Buerge et al., 2003). However, it can also produce adverse effects on human health unavoidably. Caffeine consumption during pregnancy is associated with an increased risk of fetal growth restriction (CARE Study Group, 2008). Excessive intake of caffeine can increase the risk of miscarriage, independent of pregnancy-related symptoms (Weng et al., 2008). Statistically significantly higher risk of ovarian cancer among women who reported drinking five or more cups of caffeinated coffee per day compared to non-consumers of coffee (Lueth et al., 2008). The ingestion of either a high or low glycemic index meal with caffeinated coffee significantly impairs acute insulin sensitivity compared with the effect of decaffeinated coffee (Moisey et al., 2008). Large sample and population-based studies indicated that regular daily dietary caffeine intake is associated with disturbed sleep and associated daytime sleepiness (Roehrs and Roth, 2008). For older women, blood pressure response is augmented, and subjective feelings of behavioral mood state are attenuated to a greater degree following acute caffeine ingestion (Arciero and Ormsbee, 2009). Higher level of caffeine also leads to higher risk of developing bone problems, such as osteoporosis (Hallstrom et al., 2006), alveolar bone loss in ligature-induced periodontitis (Bezerra et al., 2008), an

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d disturbing the early stages of bone healing (Duarte et al., 2009). Tea polyphenols, an excellent antioxidant extracted from tea (Panza et al., 2008), is always contaminated with amount of caffeine because of the technical constraints of decaffeination, which hampers their application in the field of pharmaceutical and functional food.

Environmentally, caffeine has been suggested as a chemical indicator of ecosystem since it is difficultly metabolized (Ogunseitan, 2002). When the exposure dosage of caffeine in water was higher than 300 mg L^{-1}, no zebrafish embryos could survive, and caffeine-treated embryos exhibited significantly reduced tactile sensitivity frequencies of touch-induced movement even when exposure dosages were very low (Chen et al., 2008). Reports also showed that caffeine is toxic to most of the aquatic organism such as *Ceriodaphnia dubia* (Moore et al., 2008) and coral algae (Pollack et al., 2009) . Therefore, release of some caffeine-containing wastes, such as coffee pulp/husk from coffee producing factory, infused tea leaves and sewage from the factory of tea polyphenols preparation, leads serious ecological pollutions and becomes a big disposal problem (Pandey et al., 2000).

To date, four major approaches for reducing caffeine content from caffeine-containing products are conventional breeding, physicochemical methods, genetic engineering and microbial degradation, whilst approach for wastes decaffeination is rarely concerned. Decaffeination through conventional breeding is difficult to achieve because of non-availability of the low-caffeine containing germplasms. Genetically-modified (GM) plants with low caffeine can be directly obtained either by down-regulating the caffeine synthesis pathway or by up-regulating the caffeine degradation pathway (Yadav and Ahuja, 2007). However, this approach can not be applied recently because of their instability and ecological risk of GM plants, even though successful study in laboratory scale was reported (Kato et al., 2000). Decaffeination with special reagents extraction (Sun et al., 2010) and chromatograph separation is usually adopted in practices (Dong et al., 2011; Lu et al., 2010; Ye et al., 2009) although these techniques need to be optimized furthermore since their lack of security or/and yield. Comparatively, decaffeination through microbial degradation is more beneficial and attractive than other methods since it can be conducted safely and at a low budgetary requirement (Gummadi and Santhosh, 2006; Ramarethinam and Rajalakshmi, 2004).

Microbial decaffeination, mainly including bacteria and fungi, was reported first in the early 1970's (Kurtzman and Schwimmer, 1971). Reports showed that *Aspergillus* and *Penicillium* (Hakil et al., 1998), *Rhizopus delemar* (Tagliari et al., 2003) and *Aspergillus tamari* (Gutierrez-Sanchez et al., 2004) can effectively degrade caffeine. Major bacteria with capability of caffeine degradation belong to *Pseudomonas* (Gokulakrishnan et al., 2005; Mazzafera, 2004; Sarath Babu et al., 2005; Dash and Gummadi, 2006a), beside some other species within the genera *Alcaligenes, Rhodococcus, Klebsiella* (Dash and Gummadi, 2006b)*, Acinetobacter, Enterobacter, Stenotrophomonas, Pantoea* spp. (Padmanabhan et al., 2003) and *Serratia marcescens* (Mazzafera et al., 1996). The characteristic and mechanism of caffeine biodegradation were investigated in many *Pseudomonas* strains which were isolated from the wastewater (Ogunseitan, 1996, 2002) and from the soils of coffee plantation area (Dash and Gummadi, 2007, 2008; Gokulakrishnan and Gummadi, 2006; Gokulakrishnan et al., 2007; Gummadi et al., 2009b) and normal agricultural land (Topp et al., 2006; Yu et al., 2008, 2009). Comparatively, the *Pseudomonas* isolated from coffee plantation area exhibits the highest caffeine tolerance, indicating that caffeine content in the growth environment might affect the caffeine tolerance capability of the strains. Recently, bioreactor for decaffeination was also developed (Gummadi et al., 2009a, 2009b) and optimized (Gummadi and Santhosh, 2010) by using *Pseudomonas* strains isolated from coffee plantation area. However, researchers never stop exploring the new strains with high caffeine tolerance and efficient caffeine degradation since the strain is the one of most important factors for developing caffeine biodegradation technique. In this study, twenty strains with high caffeine-tolerant capability were isolated from the caffeine-containing soil of tea garden, and two of them were identified as *Pseudomonas putida* based on the physiological- biochemical tests and 16S rRNA gene analysis. Effect of caffeine concentration and other culture conditions on the strain growth was also investigated.

MATERIALS AND METHODS

Preparation mediums for screening and amplifying the caffeine-tolerant bacteria

Solution with mineral substances, including KCl (0.370 g L^{-1}), $MgSO_4$ (0.205 g L^{-1}), $Fe_2(SO_4)_3$ (0.710 g L^{-1}), $Na_2HPO_4 \cdot 12H_2O$ (0.205 g L^{-1}), $CaCl_2$ (80 mg L^{-1}), $ZnSO_4 \cdot 7H_2O$ (15 g L^{-1}), $NaMoO_4 \cdot 2H_2O$ (12 g L^{-1}), $MnSO_4$ (11 g L^{-1}), $CuSO_4$ (10 g L^{-1}) and H_3BO_4 (10 g L^{-1}), was prepared and centrifuged (Beckman J2 HS; Beckman Instruments Inc., Fullerton, CA, USA) at 5000 ×g for 10 min after its pH value was adjusted to 7.0. The supernatant was collected and used for preparation of different culture mediums. Solid screening medium (SSM) for isolating the caffeine-tolerant bacteria was prepared by mixing the mineral solution with caffeine (2.5 g L^{-1}) and agar (1.5%) and autoclaved at 121°C for 10 min. Solid purifying medium (SPM) was also prepared as SSM except 20.0 g L^{-1} caffeine was supplemented. Liquid amplifying medium (LAM) was obtained after addition of caffeine (0.5 g L^{-1}) and sucrose (5.0 g L^{-1}) in the mineral solution and disinfection.

Isolation, purification and amplification of caffeine-tolerant bacteria

Soils were collected randomly from experimental tea garden of Zhejiang University. Some of them (100 g) were soaked in 1 L distilled water for 30 min. Solution containing microorganisms were

obtained by filtration and diluted 10^3-10^6 times, and spread over the SSM surface in Petri dishes (0.5 ml solution for each dish). After incubation at 30°C for 2-3 d, the fast- growing single colony was picked up with tooth tip and inoculated on SPM surface for further purification. Twenty colonies, which could grow normally on the SPM, were separately picked up again and transferred to the tubes containing 1.0 ml LAM medium (each colony for one tube), then incubated at 150 r min^{-1} and 30°C in a shaking incubator (Taicang Experimental Instruments Factory, Jiangsu, China) for amplification. Three days later, 0.3 ml of bacterial solution was mixed with 0.1 ml dimethyl sulfoxide and stored at -70°C, and the remaining solution was used for further tests.

Physiological-biochemical identification of caffeine-tolerant bacteria

Two of the above twenty strains with high-caffeine tolerant capability, recorded as CT25 and CT75, were randomly selected for physiochemical identification. The capability to metabolize carbon-containing compounds was assessed by glycolysis test, methyl red test (MR) and Voges- Proskauer test (VP), citrate utilization test, oxidase activity and starch hydrolysis test. The activity of metabolizing nitrogen-containing compounds was assessed by indole production, hydrogen sulfide production, urease production tests and gelatin liquefaction test. In order to make further determination of the objective strains, the arginine dihydrolase activity test was also performed. The identification was carried out in detail according to the "Bergey's Manual of Determinative Bacteriology" (Buchanan and Gibbons, 2000).

16S rRNA gene identification of caffeine-tolerant bacteria

Amplified caffeine-tolerant bacteria solution (0.5 ml) of strain CT25 and CT75 was centrifuged at 2000 ×g for 5 min and washed with PBS buffer, and suspended in distilled water (0.1 ml). Degenerate primers (forward primer: 5'-agagtttgatcmtggctcag; reverse primer: 5'-ggytaccttgttacgactt, predicted length 1498 bp) were used to amplify the partial 16S rRNA gene fragments. PCRs were carried out in a 25 l mixture containing 17.3 l double- distilled water, 2.5 l 10×PCR buffer, 1.5 l 25 mM $MgCl_2$, 0.5 l 10 mM dNTPs, 0.5 l each primer, 0.2 l Taq DNA polymerase (2.5 U l^{-1}) and 2 l strain resuspension. Amplification was performed on Veriti 96-well Fast Thermal Cycler (Applied Biosystems, Inc., Carlsbad, CA, USA) according to the following time program: predenaturation at 94°C for 5 min, 1 cycle; denaturation at 94°C for 45 s, annealing at 50°C for 60 s and extension at 72°C for 90 s, 30 cycles; and final extension at 72°C for 10 min, 1 cycle. Partial amplified solutions (10 l) were subjected to electrophoresis for checking presence of the target band, the remaining was sent to Invitrogen (Shanghai) Life Science Corporation for sequencing. The obtained sequences were deposited in GenBank (www.ncbi.nlm.nih.gov) after blasting with known sequences by using the tool of "blastn".

Effect of culture conditions on the growth of isolated strain

For evaluating the effect of oxygen, the amplified bacteria (50 l) of strain CT25 was inoculated in a conical flask containing 50 ml mineral solution supplemented with 2.0 g L^{-1} caffeine, and incubated in a shaking incubator at 150 r min^{-1} and 25ëC for 30- 72 h. Static culture (without shaking) was also carried out as control.

For evaluating the effect of nitrogen source, the CT25 strain was inoculated in a mineral medium supplemented with 2.0 g L^{-1} peptone instead of 2.0 g L^{-1} caffeine, and incubated at 150 r min^{-1} and 25°C for 14 to 42 h.

For evaluating the effect of caffeine concentration, the CT25 strain was incubated in mineral medium with supplement of different concentration caffeine (0.1, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 g L^{-1}) at 150 r min^{-1} and 25°C for 30 h.

For evaluating the effect of temperature, the CT25 strain was inoculated in a caffeine-supplemented (2.0 g L^{-1}) medium and shook (150 r min^{-1}) at different temperature (20, 25, 30 and 35°C) for 12-72 h.

After incubation, the optical density of bacterium solutions was measured at 600 nm (OD_{600}) with a HP8453 UV-visible spectrophotometer (Hewlett Packard, CA, USA), and the residue level of caffeine in caffeine supplemented medium was also monitored by HPLC (LC20A, Shimadzu Corporation, Tokyo, Japan) method as described previously (Liang et al., 2007).

Data analysis

Observations were made in triplicates and the mean ± standard deviation (SD) was presented. Statistics was carried out on Version 8.1 SAS System for Windows (SAS Institute Inc, Cary, NC, USA) software.

RESULTS

The isolated high caffeine-tolerant bacteria

After screening, a total of 103 colonies were obtained based on rapid proliferation on the SSM medium containing 2.5 g L^{-1} caffeine. These colonies exhibited a similar appearance, circular in shape and ivory-white in colour. Twenty strains of them could grow normally but somewhat slowly on the SPM medium supplemented with 20.0 g L^{-1} caffeine after further purification, and were recorded as CT03, CT12, CT25, CT26, CT33, CT38, CT39, CT42, CT45, CT47, CT55, CT57, CT66, CT70, CT75, CT78, CT79, CT80, CT94 and CT97 respectively. These strains can be barely differentiated from each other according to their appearances and growths, suggesting they might belong to a same genus.

The characteristics of high caffeine-tolerant bacteria

A series of determinative tests were carried out for identifying the randomly selected caffeine-tolerant strains (CT25 and CT75) because different genera have specific metabolic characteristics and different capacities to utilize amino acids, carbohydrates and proteins (Harley, 2008). Table 1 summarized the physiological-biochemical characteristics of the strains CT25 and CT75. Both CT25 and CT75 were preliminary identified as *Pseudomonas putida* after comparison with Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 2000).

The partial gene (1498bp) of 16S rRNA genes from strains of CT25 and CT75 were amplified and sequenced (Figures 1 and 2), then deposited in GenBank (GU828029 for CT25 and GU828030 for CT75).

Table 1. Physiological-biochemical characteristics of caffeine-tolerant bacteria*.

Items	CT25	CT75
Glucose fermentation	+	+
Lactose fermentation	+	+
VP & MR	-	-
Citrate utilization test	-	-
Starch hydrolysis	-	-
Oxidase	+	+
Indole production	-	-
Hydrogen sulfide production	-	-
Gelatin hydrolysis	-	-
Urease	+	+
Nitrate reduction	+	+
Arginine dihydrolase	+	+

"+" for positive and "-" for negative.

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agagtttgat catggctcag attgaacgct ggcggcaggc ctaacacatg caagtcgagc ggatgacggg agcttgctcc      80
ttgattcagc ggcggacggg tgagtaatgc ctaggaatct gcctggtagt gggggacaac gtttcgaaag gaacgctaat     160
accgcatacg tcctacggga gaaagcaggg gaccttcggg ccttgcgcta tcagatgagc ctaggtcgga ttagctagtt     240
ggtggggtaa tggctcacca aggcgacgat ccgtaactgg tctgagagga tgatcagtca cactggaact gagacacggt     320
ccagactcct acgggaggca gcagtgggga atattggaca atgggcgaaa gcctgatcca gccatgccgc gtgtgtgaag     400
aaggtcttcg gattgtaaag cactttaagt tgggaggaag ggcagtaagt taataccttg ctgttttgac gttaccgaca     480
gaataagcac cggctagctc tgtgccagca gccgcggtaa tacagagggt gcaagcgtta atcggaatta ctgggcgtaa     560
agcgcgcgta ggtggtttgt taagttggat gtgaaagccc cgggctcaac ctgggaactg catccaaaac tggcaagcta     640
gagtacggta gagggtggtg gaatttcctg tgtagcggtg aaatgcgtag atataggaag gaacaccagt ggcgaaggcg     720
accacctgga ctgatactga cactgaggtg cgaaagcgtg gggagcaaac aggattagat accctggtag tccacgccgt     800
aaacgatgtc aactagccgt tggaatcctt gagattttag tggcgcagct aacgcattaa gttgaccgcc tggggagtac     880
ggccgcaagg ttaaaactca aatgaattga cgggggcccg cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg     960
aagaacctta ccaggccttg acatgcagag aactttccag agatggattg gtgccttcgg gaactctgac acaggtgctg    1040
catggctgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgtaac gagcgcaacc cttgtcctta gttaccagca    1120
cgttatggtg ggcactctaa ggagactgcc ggtgacaaac cggaggaagg tggggatgac gtcaagtcat catggccctt    1200
acggcctggg ctacacacgt gctacaatgg tcggtacaga gggttgccaa gccgcgaggt ggagctaatc tcacaaaacc    1280
gatcgtagtc cggatcgcag tctgcaactc gactgcgtga agtcggaatc gctagtaatc gcgaatcaga atgtcgcggt    1360
gaatacgttc ccgggccttg tacacaccgc ccgtcacacc atgggagtgg gttgcaccag aagtagctag tctaaccttc    1440
gggaggacgg ttaccacggt gtgattcatg actggggtga agtcgtaaca aggtagcc                            1498
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Figure 1. Partial sequence of 16S rRNA gene from CT25 strains (GU828029).

Homology search with the tool of "blastn" showed that the sequences of 16S rRNA gene from CT25 and CT75 were 99% identical with corresponding sequences of *Pseudomonas putida* (Table 2). The result confirmed the previous identification on the basis of physiological-biochemical characteristics, therefore, the isolated twenty strains, including randomly selected CT25 and CT75, were the *Pseudomonas putida* strains with high caffeine-tolerant capability.

The growth of high caffeine-tolerant bacteria

Incubation results showed that the OD_{600} value of CT25 strain under shaking culture condition was significantly higher than that undern static culture condition (Table 3), suggesting the strain CT25 is an aerobe and its growth depends obviously on oxygen concentration in medium.

Different source of N and C test showed that the OD_{600} value of CT25 cultured in mineral medium with addition of

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agagtttgat catggctcag attgaacgct ggcggcaggc ctaacacatg caagtcgagc ggatgacggg agcttgctcc      80
ttgattcagc ggcggacggg tgagtaatgc ctaggaatct gcctggtagt gggggacaac gtttcgaaag gaacgctaat     160
accgcatacg tcctacggga gaaagcaggg gaccttcggg ccttgcgcta tcagatgagc ctaggtcgga ttagctagtt     240
ggtggggtaa tggctcacca aggcgacgat ccgtaactgg tctgagagga tgatcagtca cactggaact gagacacggt     320
ccagactcct acgggaggca gcagtgggga atattggaca atgggcgaaa gcctgatcca gccatgccgc gtgtgtgaag     400
aaggtcttcg gattgtaaag cactttaagt tgggaggaag ggcagtaagt taataccttg ctgttttgac gttaccgaca     480
gaataagcac cggctaactc tgtgccagca gccgcggtaa tacagagggt gcaagcgtta atcggaatta ctgggcgtaa     560
agcgcgcgta ggtggtttgt taagttggat gtgaaagccc cgggctcaac ctgggaactg catccaaaac tggcaagcta     640
gagtacggta gagggtggtg gaatttcctg tgtagcggtg aaatgcgtag atataggaag gaacaccagt ggcgaaggcg     720
accacctgga ctgatactga cactgaggtg cgaaagcgtg gggagcaaac aggattagat accctggtag tccacgccgt     800
aaacgatgtc aactagccgt tggaatcctt gagattttag tggcgcagct aacgcattaa gttgaccgcc tggggagtac     880
ggccgcaagg ttaaaactca aatgaattga cgggggcccg cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg     960
aagaacctta ccaggccttg acatgcagag aactttccag agatggattg gtgccttcgg gaactctgac acaggtgctg    1040
catggctgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgtaac gagcgcaacc cttgtcctta gttaccagca    1120
cgttatggtg ggcactctaa ggagactgcc ggtgacaaac cggaggaagg tggggatgac gtcaagtcat catggccctt    1200
acggcctggg ctacacacgt gctacaatgg tcggtacaga gggttgccaa gccgcgaggt ggagctaatc tcacaaaacc    1280
gatcgtagtc cggatcgcag tctgcaactc gactgcgtga agtcggaatc gctagtaatc gcgaatcaga atgtcgcggt    1360
gaatacgttc ccgggccttg tacacaccgc ccgtcacacc atgggagtgg gttgcaccag aagtagctag tctaaccttc    1440
gggaggacgg ttaccacggt gtgattcatg actggggtga agtcgtaaca aggtagcc                            1498
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Figure 2. Partial sequence of 16S rRNA gene from CT75 strains (GU828030).

Table 2. Blast result of 16S rRNA gene from CT25 and CT75.

Accession	Description	E-Value	Consistency (%)
GU828029	*Pseudomonas putida* strain 25 16S ribosomal RNA gene, partial sequence	Sequence from CT25	
GU828030	*Pseudomonas putida* strain 75 16S ribosomal RNA gene, partial sequence	Sequence from CT75	
CP002290	*Pseudomonas putida* BIRD-1, complete genome	0.0	99
CP000926	*Pseudomonas putida* GB-1, complete genome	0.0	99
AE015451	*Pseudomonas putida* KT2440 complete genome	0.0	99
CP000712	*Pseudomonas putida* F1, complete genome	0.0	99
AM411058	*Pseudomonas putida* partial 16S rRNA gene, strain 5zhy	0.0	99
DQ657850	*Pseudomonas* sp. ND9 16S ribosomal RNA gene, partial sequence	0.0	99

Table 3. Effect of oxygen on growth of CT25 in the medium with 2.0gL^{-1} caffeine*.

Items	30 h	72 h
Static culture	0.0633±0.0034 a	0.0634±0.0067 a
Shaking culture	0.1461±0.0032 b	0.2827±0.0029 b

*The OD_{600} was used as growth indicator of bacterium, and the different letters in column indicated significant difference at $p<0.05$.

2.0 g L^{-1} peptone for 14 h was 28.7 times as high as that in medium supplemented with 2.0 g L^{-1} caffeine, and when the culture time was prolonged furthermore, the ratio of OD_{600} between the two different sources decreased significantly, being 6.9 times for 30 h and 5.5 times for 42 h (Table 4). It was clear that peptone hastens the growth of CT25, whilst caffeine lowers the initial growth rate and prolongs the amplification time to achieve the plateau phase.

The growth of strain CT25 was accelerated when the caffeine concentration increased from 0.1 to 0.5 g L^{-1} in the medium, and inhibited gradually when the caffeine concentration increased furthermore (Table 5). Although the CT25 could tolerate high concentration of caffeine up to 20 g L^{-1}, it multiplied slowly, indicating the high concentration of caffeine may inhibit the growth of CT25.

The test of incubation temperature showed that growth

Table 4. Comparison of CT25 growth cultured with different sources for nitrogen and carbon*.

Nitrogen source	14 h	30 h	42 h
Caffeine (2.0g L^{-1})	0.0305±0.0012a	0.1461±0.0032a	0.2514±0.0033a
Peptone (2.0g L^{-1})	0.8911±0.0045b	1.0084±0.0049b	1.3896±0.0174b

* The OD_{600} was used as growth indicator of bacterium, and the different letters in column indicated significant difference at $p<0.05$.

Table 5. CT25 growth in the mediums with addition of different caffeine*.

Caffeine concentration (g L^{-1})	OD_{600}
0.1	0.1425±0.0021d
0.5	0.1788±0.0076e
1.0	0.1693±0.0049e
2.0	0.1468±0.0085d
5.0	0.1313±0.0058c
10.0	0.1013±0.0037b
20.0	0.0422±0.0013a

* Incubation time was 30 h, and the different letters in column indicated significant difference at $p<0.05$.

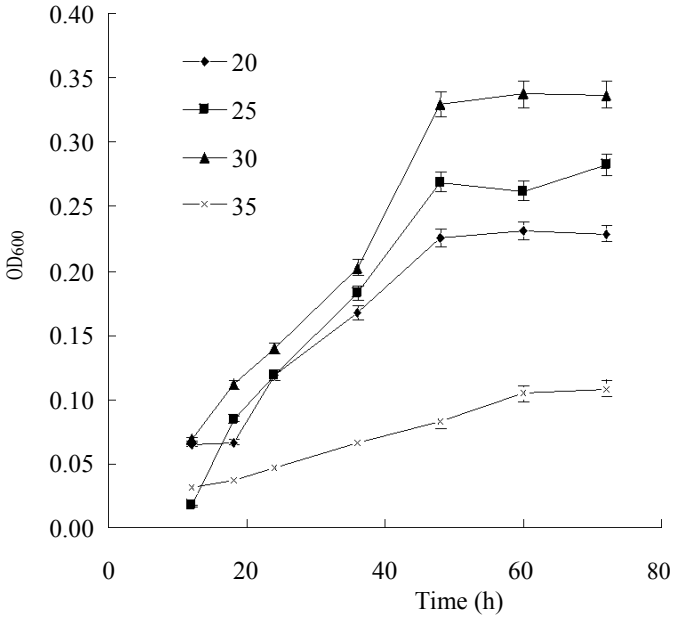

Figure 3. The effect of temperature on growth of CT25 cultured in medium supplemented with 2.0 g L^{-1} caffeine. The bar shows the standard deviation.

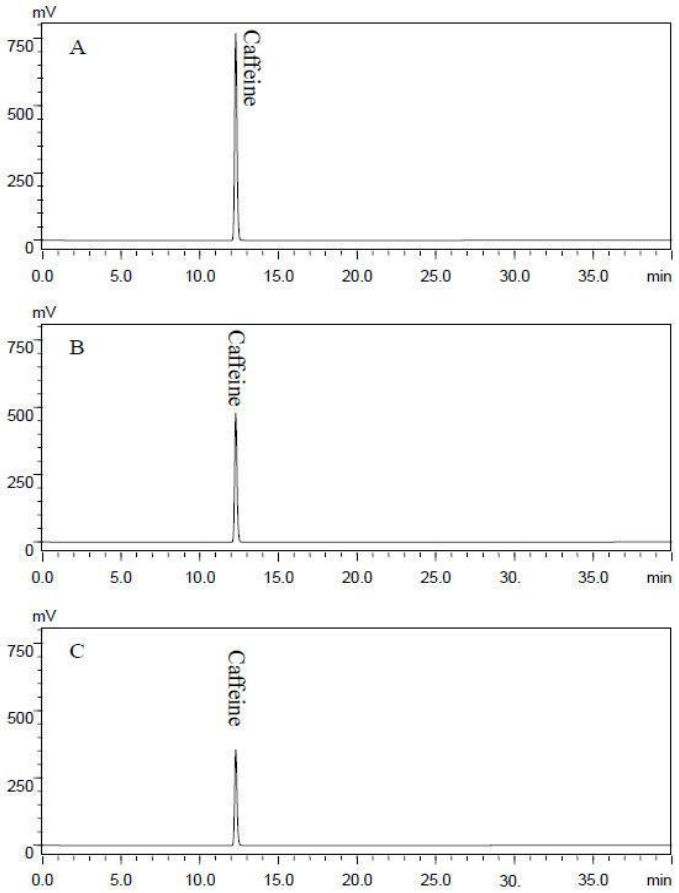

Figure 4. The HPLC chromatograph of mediums supplemented with 2.0 g L^{-1} caffeine before and after incubation. Prior to analysis all the mediums were diluted 10 times and filtrated through 0.22 m microporous membrane. A, before incubation; B, after incubation at 35ëC for 72 h and C, after incubation at 30°C for 72 h.

strain CT25 increased remarkably when ambient temperature increased from 20 to 30°C, and decreased significantly when the temperature increased up to 35°C (Figure 3). The result also showed that the early 48 h was the time phase for rapid amplification of strain when the temperature ranged from 20 to 30°C. After incubation for 48 h, the OD_{600} of the strain cultured at 35°C accounted for only 36.68% of that at 20°C, 30.73% of that at 25°C and 25.07% of that at 30°C, suggesting that the most favorable incubation temperature for the CT25 is around 30°C.

HPLC analysis showed that caffeine residue in medium decreased along with an increase in incubation time, and around 50% of initial caffeine was degraded by the CT25 cultured at 30°C for 72 h whilst less than 20% at 35°C (Figures 4 and 5).

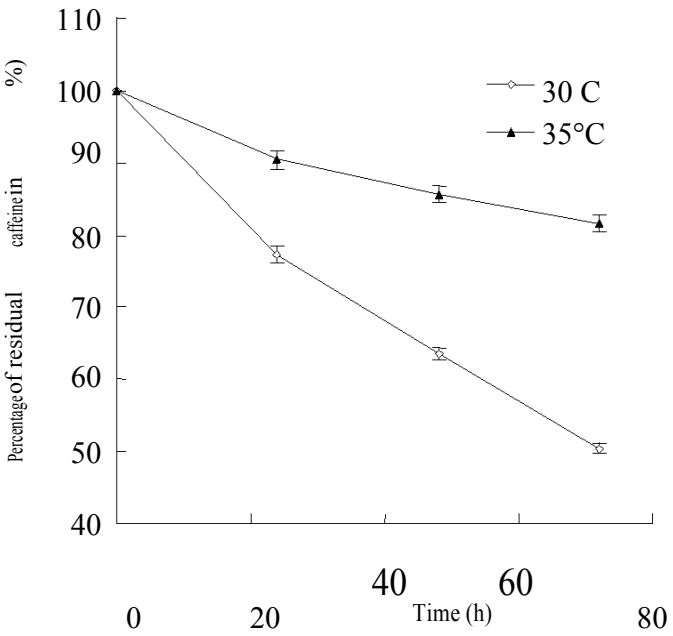

Figure 5. Caffeine- degrading efficiency of CT25 cultured in medium supplemented with 2.0 g L-1 initial caffeine at different temperature. The bar shows the standard deviation.

DISCUSSION

The isolated twenty strains could grow normally on the medium supplemented with 20 g L^{-1} caffeine, indicating these strains can tolerate high concentration of caffeine and metabolize it as sole nitrogen (N) and carbon (C) for their growth demand since no other source of N and C was present in that medium. Previous reports showed that some *Pseudomonas* sp. strains isolated from coffee plantation area could survive in the mediums supplemented with caffeine at initial concentration of 10 g L^{-1} (Gokulakrishnan and Gummadi, 2006; Gummadi and Santhosh, 2006; Dash and Gummadi, 2008), 15 g L^{-1} (Dash and Gummadi, 2007; Dash et al., 2008) and 20 g L^{-1} (Gummadi et al., 2009b; Gummadi and Santhosh, 2010) and degrade the caffeine sufficiently in short period. From this view, the isolated strains in this study might possess similar high caffeine-tolerant capability to the reported ones although some investigation should be further conducted with these strains. It was also suggested that soil microorganisms have the ability for natural enrichment under different ecological condition according to their chemotaxis (Mazzafera, 2002). High caffeine-tolerant bacteria can be isolated from caffeine-containing soils such as tea and coffee garden where caffeine is continuously released from the litters of these plants.

Series physiological-biochemical reactions (Buchanan and Gibbons, 2000) and the sequence of 16S rRNA gene (Garrity et al., 2004) are widely used for bacterial identification. The randomly selected strains (CT25 and CT75) with high caffeine-tolerant capability were identified as *Pseudomonas putida* according to the similarity of physiochemical characteristics and 16S rRNA gene sequences. It seemed that all of the isolated twenty strains might belong to *Pseudomonas putida* since the appearance of the colonies was quite similar, and this species might be a dominant bacterium in soil of tea garden because special metabolic pathway(s) related to caffeine degradation were reported to be present inherently in many *Pseudomonas* strains (Dash and Gummadi, 2006b; Mazzafera, 2004; Yamaoka-Yano and Mazzafera, 1999; Yu et al., 2009).

The growth of strain CT25 was highly depended on oxygen in liquid medium, which is consistent with other reports on the growth of *Pseudomonas* strains (Beltrán et al., 2006; Gummadi and Santhosh, 2006; Gummadi et al., 2009b; Middelhoven and Lommen, 1984; Woolfolk, 1975). Although caffeine could be used as sole source for N and C, the strain CT25 amplified more slowly in medium supplemented with caffeine than that with peptone, and its growth was significantly inhibited especially when high level caffeine was supplemented. Similar results were reported previously in the studies about other caffeine-degrading strains (Sarath Babu et al., 2005; Gokulakrishnan and Gummadi, 2006; Dash and Gummadi, 2007, 2008; Gummadi et al., 2009b). It was suggested that the isolated bacteria can utilize the caffeine as their nonpreferential N and C sources and may take time to activate their metabolic pathway(s) to transform the caffeine into some components directly used for growth. This result agrees with some findings that the caffeine-degrading enzymes of bacteria were induced (Dash and Gummadi, 2007; Gummadi and Santhosh, 2006; Sarath Babu et al., 2005; Ogunseitan, 2002; Woolfolk, 1975). The fastest amplification was observed when the strain CT25 was incubated at around 30°C, while amplification would dramatically decrease when the strain was incubated at 35°C, which coincides with the previous observations on the growth of other *Pseudomonas* strains (Dash and Gummadi, 2007; Sarath Babu et al., 2005) and the optimum catalytic temperature of caffeine-degrading enzyme (Beltrán et al., 2006; Yamaoka-Yano and Mazzafera, 1999). However, this result is not in accordance with another report in which the optimum temperature for the growth of *Pseudomonas* sp. strain was 35°C (Gummadi and Santhosh, 2006). The difference might be explained as different strain or other culture parameters such as caffeine concentration.

Caffeine was metabolized rapidly when the CT25 was incubated at 30°C, while slowly at 35°C (Figures 4 and 5), indicating that the caffeine degradation was positively correlated with the bacterial growth as more bacterial cells would metabolize much more caffeine into nutrition for further bacterial growth. Thus, in order to improve the decaffeination efficiency, the initial inoculums concentration should be optimized. Compared with the caffeine residue, metabolites of caffeine were barely detected in the incubated medium (Figure 4), suggesting that initial degradation of caffeine might be a rate-limiting step and

the metabolites might be instantly broken down furthermore.

The bacteria isolated from soil of tea garden (such as CT25) have high capability for caffeine- tolerance and caffeine-degradation, and can be used as an alternative for decaffeination of tea products or caffeine-containing wastes. Further studies like usage method (immobilization or not), optimization of decaffeination efficiency and assessment of products quality and security, should be conducted prior to exploitation of the bacterial strains for caffeine degradation purpose.

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