Journal of Sciences, Islamic Republic of Iran 24(4): 305 - 312 (2013) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Biodecaffeination by *Pseudomonas pseudoalcaligenes* TPS8, an Isolated Strain from Tea Plantation Soil

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Received: 28 September 2013 / Revised: 9 November 2013 / Accepted: 1 January 2014

Abstract

Development of an environmental friendly and cost-effective process for microbial degradation of caffeine to non-toxic compounds are promising to solve the problems of physiochemical extraction of caffeine in the treatment the caffeine containing agro-industrial effluents. Thirteen bacterial strains, isolated from tea plantation soils in the north region of Iran, were screened to show their abilities in using caffeine as the sole source of carbon and nitrogen. The intrinsic tolerance of the isolated strains to the caffeine substrate was measured in a defined and complex medium by using the agar dilution method. Based on the tolerance efficiency, isolate TPS8 which showed maximum tolerance to caffeine was selected and identified as *Pseudomonas pseudoalcaligenes* strain TPS8 (GenBank accession number KF414528) according to the cultural and physiochemical characteristics and also 16S rDNA gene sequencing. Growing cells of *P. pseudoalcaligenes* TPS8 were used for the biodecaffeination experiments. The maximum removal of caffeine (80.2%) was reached after a 72 h incubation using 2.5 g/l of caffeine substrate without further optimization. Our results show that growing cells of *P. pseudoalcaligenease* TPS8 can thus be efficiently used as a simple and cheap process for preparative decaffeination from agro-industrial effluents. The present survey is the first report on biodecaffeination using *Pseudomonas pseudoalcaligenes*.

Keywords: Caffeine; Microbial degradation; Tolerance profile*; Pseudomonas pseudoalcaligenes* strain TPS8

Introduction

Caffeine (C8H10N4O2) is a purine alkaloid present in the beans, leaves and fruits of more than sixty plant species, such as tea (*Camellia* species), coffee (*Coffea* species), cocoa (*Theobroma cacao*) and the like [14]. Caffeine is widely used in pharmaceutical preparations and beverage products and can be associated with a variety of pharmacological effects. It is used as a cardiac, neurological and respiratory stimulant and also as a diuretic compound [7]. Caffeine effects on sleep, but these effects are different in all individuals. Caffeine rises alertness during awakeness cycle, However, consumption on Caffein might lead to adisorder called

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Insomnia. [28]. The higher level of caffeine leads to higher risk of health problems such as adrenal stimulation, irregular muscular activity, cardiac arrhythmias, osteoporosis and heart output enhancement [13]. Excess caffeine is reported to cause mutation, inhibition of DNA repairs and inhibition of adenosinemonophosphodiesterase [26, 30] and causes of miscarriage in pregnancy period [9]. Regardless of the health effects, decaffeination process is important from the view of environmental preservation [23]. Influx of coffee processing industrial effluents into lakes can give rise to drinking water to be unsuitable [3]. Caffeine in soil also restrains seed grain germination and its growth [1]. Several methods including organic solvents, water diffusion and super critical carbon dioxide extraction have been applied to the removal of caffeine [22]. Considering, these procedures are hazardous to health, expensive, time consuming, intensive works and non-specific for the removal of caffeine, alternative routes for its removal including microbial biodecaffeination are being constantly explored. Caffeine is regarded as toxic for many microorganisms at a concentration of 1 g/l. However, caffeine degradation has been reported for a variety of microbial species belonging to *Penicillium* spp. [2, 27], *Thermomonospora* sp. [29], *Serratia* sp. [18], *Klesiella* and *Rhodococcus* sp. [17], *Alcaligenes* spp. [19], *Trichosporon* sp. [15], *Pseudomonas* spp. [6, 8, 10, 11, 25, 31, 34], *Brevibacterium* sp. [20], *Aspergillus* spp. [12,21], *Chrysosporium keratinophilum* [21]. We would like to link this study to isolate and characterize native bacterial strains to high-tolerance ability of Caffeine and a potential for efficient caffeine degradation under growing-cell conditions. We screened 13 different bacteria strains for their abilities and hereby using caffeine as the sole source of carbon and nitrogen. Finally, a newly isolated strain of *Pseudomonas pseudoalcaligenes*, designated as TPS8, with high tolerance pattern as well as an efficient caffeine degradation was showed over our experiments. The present investigation gives the first evidence for the biodecaffeination by *P. Pseudoalcaligenes*.

Materials and Methods

Chemicals and Media

Caffeine (> 99%) used for caffeine degradation experiments was from Sigma-Aldrich. The caffeine stock solution was prepared in distilled water and maintained at 4º C following sterilization through a 0.22-µm microbiological filter. Nutrient agar (3 g/l beef extract, 5 g/l peptone and 20 g/l agar pH 7.4) was purchased from Kardanazma, Iran. All other chemicals are of analytical grade.

Enrichment cultures and Isolation of caffeine-degrading bacteria

Samples were collected randomly from tea plantation soils obtained in different areas of Northern Iran and stored in the dark at 4º C until use. Enrichment culture was carried out by mixing one gram of collecting soil sample with 100 ml of autoclaved mineral M9 medium from Sambrook et al. [24] (7.5 g/l $Na_2HPO_4.2H_2O$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 0.5 g/l $MgSO_4.7H_2O$ and 0.015 g/l $CaCl_2$ pH 7± 0.1) supplemented with 1 g/l of caffeine as the sole source carbon and nitrogen sources in 250 ml Erlenmeyer flask and incubated at 28º C in a rotary shaker set at 150 rpm for 7 days. The initial pH of the media was adjusted to 7. After enrichment, single colonies were isolated on minimal M9-caffeine agar media by spread plate method and subjected to further purification by streak plate method on the same media. Morphological different colonies were selected as inocula for determining their caffeine tolerance profile using the agar dilution method of Washington and Sutter [33].

Caffeine tolerance the isolated bacterial strains

For the caffeine tolerance profiles, 30 ml of melted Nutrient agar and Modified M9 defined minimal salt (glucose 2g/l; NH_4Cl 1g/l; $MgSO_4.7H_2O$ 0.5 g/l; $CaCl_2$ 0.015 g/l; $FeSO_4.7H_2O$ 0.03 g/l; NaCl 0.5 g/l and Phosphate buffer 0.1 M pH 7.4) agar media supplemented with different concentrations of caffeine ranges from 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 g/l were prepared and poured into 10 cm plates. Each plate was subdivided into eight zones and 20 µl of 0.5 McFarland standard of bacterial suspension (1.5×10^8 cfu/ml), prepared from bacteria cultures grown overnight for 24 h, was transferred on each plate using a sampler followed by incubation at 28º C for up to 5 days. The growth of bacteria strains was calculated visually. Each plate was run in triplicates. The colony with the highest caffeine tolerance ability was designated as TPS8 and selected for further investigation.

Identification of the isolate TPS8

The isolate TPS8 is characterized by physiological-cultural and biochemical tests with 16S rDNA gene sequence analysis. Morphological and physiological characteristics were performed according to the "Cowan and Steel" Manual for the Identification of Medical Bacteria [5]. The tests included gram's reaction, colony shape, color on nutrient agar, presence of oxidase and catalase, motility, hydrolysis of tween 80, utilization of citrate (Simmons method), tyrosine hydrolysis, urease

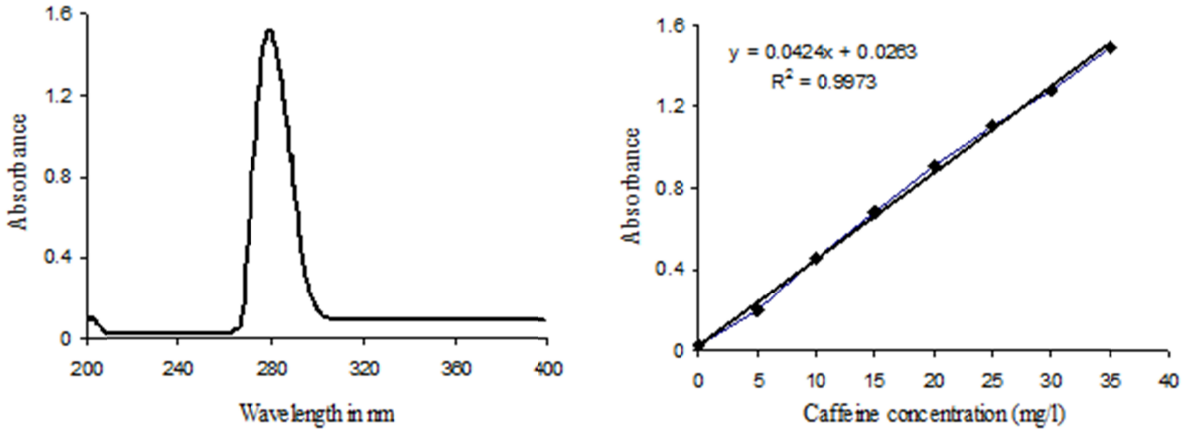

Figure 1. UV/vis absorption spectrum (A) and construction of a calibration curve (B) for determination of the concentration of caffeine in caffeine containing solutions by UV Analysis.

production, H_2S production, nitrate reduction, casein hydrolysis, lecithinase production, gelatin liquefaction, and the production of acid from sugars and growth of 42º C. The isolate was further characterized based on molecular phylogenetic analysis as described below. Genomic DNA was extracted from colonies using a Genelute DNA extraction kit (Sigma) by following the recommended procedure of the manufacturer. Universal primers 8F (5′-agagtttgatcctggctcag-3′) and 1541R (5′-aaggaggtgatccagccgca-3′) were used to amplify the partial 16S rDNA gene [16]. The PCR reaction was performed in mixture contained 0.6 µl of Smar –Taq Pfu DNA polymerase (5 U/ µl); 5 µl PCR buffer (20 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 5 mM 2ME, added Stabilizer, glycerin); 1 µl of each primer (25 µM); 4 µl $MgCl_2$ (50 mM); 0.6 µl dNTPs (10 mM) and 2 µl DNA template (180 ng/ µl) in a final volume of 50 µl with a thermal cycler (Eppendorf, Germany). Amplification was performed according to the following time program: 94°C for 10 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, and a final extension step at 72°C for 10 min. Amplified PCR products (5 µl) were subjected to standard gel electrophoresis by using 1% agarose gel and 1XTAE buffer with a 1kb DNA ladder (Fermentase) as a molecular weight marker. The gel-purified PCR product was sent to Macrogen Company (Seoul, Korea) for sequencing in directions with an automated sequencer. The sequences of 16S RDNA of the isolate were deposited in GenBank after analyzing with registered sequences by using the tool of BLASTN (http://www.ncbi.nlm.nih.gov/blast). Phylogenetic analysis was performed with Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 by using the Neighbor-Joining method [32].

Caffeine degradation experiments

A loop full of grown culture of *P. Pseudoalcaligenes* TPS8 on nutrient agar medium fortified with 1 g/l caffeine transferred to 250-ml Erlenmeyer flasks containing 50-ml of mineral M9 medium [24] supplemented with various concentrations of caffeine (0.5-5 g/l) as the sole source carbon and nitrogen sources and incubated at 28º C for 120 h under agitation 150 rpm on a rotary shaker. Time-course samples were withdrawn at different intervals of time and subjected them to analysis of cell growth and residual caffeine in a UV–Vis spectrophotometer (Specord 210, Carel Zeiss Technology, Germany). The cell mass concentrations were determined from triplicate 2 ml samples, which were centrifuged for 10 min at 5000 rpm. The pellets were washed with distilled water, recentrifuged, dried at 75º C for 36 hours and weighted [4]. Caffeine degradation in the culture media was monitored by a decrease in absorbance in a UV- visible Analytik Jena's spectrophotometer with quartz cuvettes of 2 cm optical path according to the Lakshmi and Das [15] (Fig. 1A). The cells were centrifuged at 8000 rpm for 10 min at 4 C and the supernatants were used for determination of the residual caffeine in the cultures. Percentage of caffeine removal was calculated as follows: caffeine removal (%) = [(initial caffeine concentration- residual caffeine concentration) / (initial caffeine concentration)] × 100 [31]. Calibrating solutions were prepared by diluting standard solutions to the following caffeine concentration interval 5 up to 35 mg/l. Good linearity for the concentration interval examined by the equation and the coefficient of determination for the calibration plot as shown in Fig.1B. All experiments were performed in triplicate and the means of three separate experiments with the standard deviation shown by

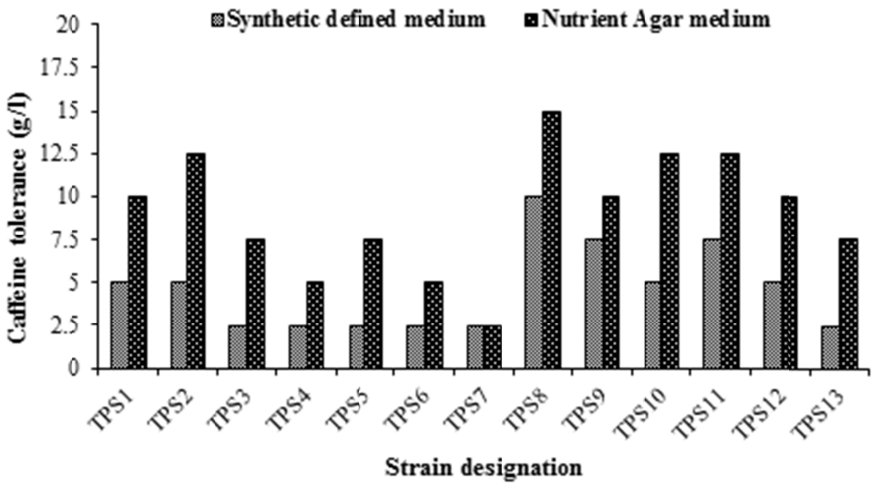

Figure 2. Caffeine tolerance profile of bacterial strains.

vertical bars.

Results

Screening for high caffeine-tolerant bacteria

A selective screening method based on enrichment technique was adapted for the selection of bacterial cultures on the basis of growing on caffeine in a well-defined liquid media as described in Materials and methods section. Thirteen bacterial strains were isolated from collecting soil samples of tea plantations (northern Iran), which were designated as isolates TPS1-TPS13. In order to reach efficient biodecaffeination, it is needed to apply an efficient bacterial strain which can tolerate high caffeine concentrations. Keeping in view, caffeine was added to nutrient agar and synthetic defined minimal salt media at concentration ranging from 2.5 to 20 g/l and the intrinsic tolerance of the bacterial strains to caffeine was evaluated (Fig. 2). It is clear from the results that isolate TPS8 have maximum caffeine tolerances with a tolerance pattern 10 and 15 g/l in the defined and complex media, respectively (Fig. 2). Because of its maximum tolerance, strain TPS8 was selected, characterized and examined for biodecaffeination experiments.

Characteristics of the strain TPS8 with potential caffeine degradation

The isolate TPS8 identified by combining its morphological and biochemical characteristics with information obtained from its 16S rDNA gene sequences. Strain TPS8 was shown to be a Gram-negative, non-sporulating, strictly aerobic bacilli, and produced catalase and oxidase. The colonies appeared round, smooth and not formed a pigment. On the basis of cultural and morphological characteristics as well as physiochemical characteristics, the isolate is preliminary placed in the genus of the *Pseudomonas*. Microbial characteristics of strain SHL1 as studied are listed in Table 1. The approximate full-length 16S rDNA gene (1502 pb) from strain TPS8 was amplified and sequenced. 16S RDNA gene sequences of strain TPS8

Table 1. Phenotypic characteristics of caffeine-degrading strain TPS8

Characteristics	Strain TPS8
Shape	Rod
Gram reaction	Gram-negative
Oxidase	Positive
Catalase	Positive
Nitrate reduced	Positive
Simmons citrate	Positive
Urease	Negative
Gelatinase production	Negative
H_2S from TSI	Negative
Casein hydrolysis	Negative
Tween 80 hydrolysis	Negative
Tyrosine hydrolysis	Positive
Lecithinase production	Negative
Acid production from:	
glucose	Negative
arabinose	Negative
fructose	Negative
maltose	Negative
manitol	Negative
sucrose	Negative
xylose	Negative
ethanol	Positive
glycerol	Positive
Growth in 42º C	Positive

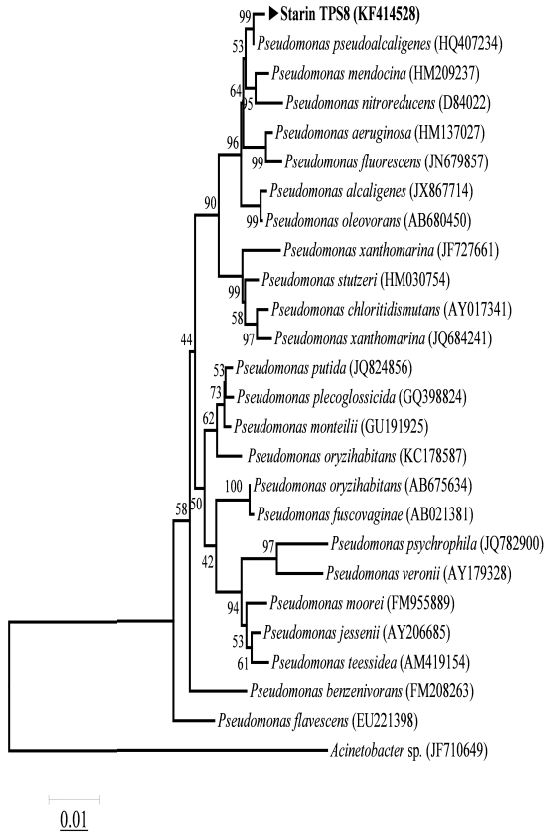

Figure 3. Phylogenetic tree of strain TPS8 and the related taxa based on amplification of 16S rDNA gene. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. *Acinetobacter* sp. was used as outgroup. Scale bar represents 0.01 substitutions per nucleotide position. GenBank accession numbers are given in parentheses.

were deposited in the GenBank and accession number is KF414528. Phylogenetic analysis implied the strain was a member of genus of *Pseudomonas* sp., and the closest phylogenetic relative was *P. pseudoalcaligenes* (Gene Bank accession no HQ407234; identity over 99%). In Figure 3, Phylogenetic tree obtained by neighbor-joining analysis of 16S rDNA gene sequences shows the position of the strain TPS8 within the genus *Pseudomonas*. Based on these obtained results, the isolate was identified as *Pseudomonas pseudoalcaligenes* strain TPS8.

Results of biodecaffeination experiments

Pseudomonas pseudoalcaligenes strain TPS8 grew in M9 broth medium when caffeine at the initial concentrations 0.5 to 5 g/l was provided as the sole carbon and nitrogen sources. The time course of caffeine removal by *P. pseudoalcaligenes* TPS8 was monitored during 144 h incubation period (Fig. 4A and B). As shown by the determination of the dry biomass levels at different caffeine supplementation (Fig. 4A), the growth of TPS8 was increased (cell dry weight 0.51 g/l) when the initial caffeine concentration increased to 2.5 g/l along with a rise incubation time for 72 h. However, higher amounts of caffeine gradually decreased the growth of strain TPS8, probably because of caffeine inhibition [10]. When the strain grew to the late-exponential of growth phase (72 h growth with cell dry weight 0.51 g/l), maximum caffeine removal (80.2%, from 2.5 g/l to 0.495 g/l) was attained and decreased significantly when the strain entered in the stationary phase. During these periods, the caffeine removal percentage in the medium decreased to 76.5%, 78.2% and 77.7% after 96, 120 and 144 h, respectively (Fig. 4B).

Discussion

Attempts for the removal toxic caffeine from the polluted effluents through chemical and microbiological processes have been tried. The advantage of microbial methods is that caffeine removal from the polluted environments is so fast and improves the nutritional value of the coffee pulp. Due to high consumption of caffeine and related methylxanthines present in foods, beverages pharmaceutical and agronomy industries, this is not much surprising that some caffeine-degrading microorganisms have been screened for in the environment. The initial reports on the caffeine degradation by microorganisms were in early 1970s [27]. Caffeine degradation is catalyzed by several microorganisms with potential of caffeine degradaing belonged to bacterial and fungal species. In bacteria, *Pseudomonas* species and in fungi *Aspergillus* and *penicillium* species are efficient in removal of caffeine. In the current study, enrichment of the caffeine-degrading bacteria were performed by adding soil samples, obtained from different areas of the north part of Iran, into M9 mineral salts medium containing 1 g/l caffeine as the sole source of carbon and nitrogen. 13 Colonies with different kind of morphology were isolated from M9-caffeine agar plates. Among them, strain TPS8 has been shown to have the tremendous caffeine tolerance ability and a potential to degrade this compound under ambient condition. By combining morphological and physiological characteristics with 16S rDNA gene sequences analysis, this isolate was identified as *Pseudomonas pseudoalcaligenes* strain TPS8 (GeneBank accession no. KF414528). This report presents data for the first time on ability of the novel

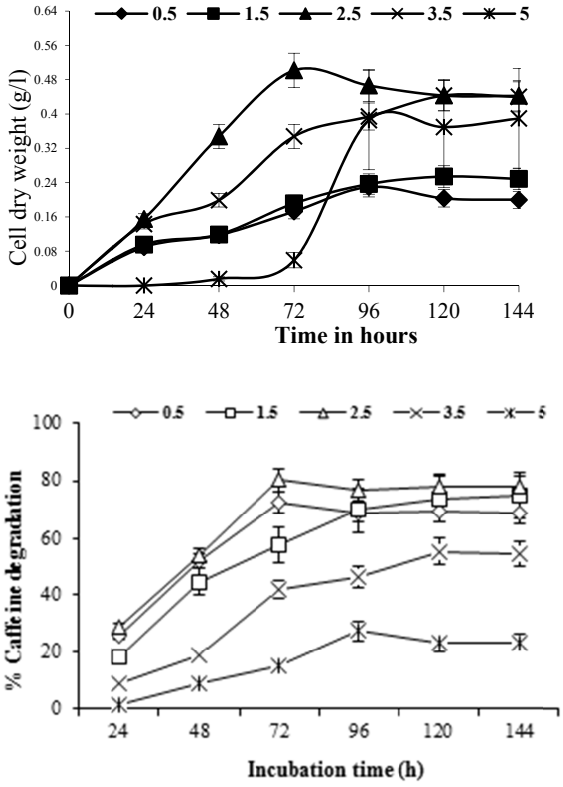

Figure 4. Time course of caffeine degradation by *P. pseudoalcaligenes* TPS8 (A) Curve growth of strain TPS8 in the MM9 medium with addition of different caffeine concentrations. (B) Effect of initial caffeine concentration on its degradation by strain TPS8 in the MM9 medium. The results represent the means of three separate experiments, and deviation bars indicated.

isolated strain *Pseudomonas pseudoalcaligenes* TPS8 in the development of an environmental friendly process for microbial caffeine degradation. The *P. pseudoalcaligenes* TPS8 being reported here showed 80.2% of 2.5 g/l caffeine removed in 72 hours incubation without further optimization. The first caffeine biodegradation was reported with strains of *Penicillium roqueforti* and *Stemphylum* sp. (27). But, the strains were capable of caffeine degrading in concentration of 0.19 g/l after 29 h. Mazzafera and co-workers (18) developed a process for caffeine degradation using *Serratia marcescens* which is able to degrade 100% of 0.6 g/l of caffeine after 72 h. The induced bacterial cells of *Pseudomonas alcaligenes* CFR1708 were found to be capable of completely degrading caffeine (1 g/l) from solutions containing caffeine in 6 h (25). Gokulakrishnan et al. (11) reported the biodegradation of caffeine by *Pseudomonas* sp. strain GSC1182 which showed 80% degradation of caffeine in 48 h when caffeine was used as the sole carbon and nitrogen source. A strain of *Pseudomonas stutzeri* Gr21ZF has been reported for caffeine degradation which is able to degrade 59% of caffeine at concentration of 1.2 g/l after 24 h (8). Gutiérrez-Sánchez et al. developed a process for studies of caffeine degradation with *Aspergillus tamari*. At a range of 2 to 4 g/l of initial caffeine concentrations, after 96 h of fermentation, 41-51 % of the initial caffeine was degraded (12). There are many attempts to improve the caffeine degradation using optimization processes by varying the environmental parameters and nutrient condition (addition of external carbon and nitrogen sources for example). Madyastha and Sridhar (17) reported a process optimization for caffeine degradation with *Klebsiella* and *Rhodococcus* sp. by adding glucose and reported 100% caffeine degradation after 10 h of incubation time with initial caffeine concentration of 0.5 g/l. Syed Baker and co-workers (31) reported that induced cells of a strain of *Pseudomonas* sp. S7, isolated from *Coffee Arabica* L, showed decaffeination of 98.61% under optimization process by varying the environmental parameters such as pH, shaking speed and time incubation. El-Mched et al. (8) developed a process optimization for caffeine degradation with *Pseudomonas stutzeri* Gr21ZF in the presence of sucrose (5 g/l) which 80.1% caffeine degradation was obtained in 48 hrs. The caffeine removal by isolated strain *P. pseudoalcaligenes* TPS8 was comparable to those previously reported for caffeine degradation (Tabel 2). Compared with previous reports, our results show that growing cells of *P. pseudoalcaligenes* TPS8 can thus be efficiently used as a simple, safe and cheap process for preparative decaffeination from industrial effluents. It should be highlighted that this yield (80.2%) was achieved without further optimization. This result clearly suggested the great potential of the isolate TPS8 for microbial degradation of caffeine.

Conclusion

We have described here the details of the screening native Iranian bacterial strains in being able to perform biodecaffeination process. A novel isolated strain, *Pseudomonas pseudoalcaligenes* TPS8, was screened from tea plantation soil, which has high ability for caffeine tolerance (up to 15g/l) and caffeine degradation (80.2 %) without any external carbon/nitrogen source addition and further process optimization, which increase the cost of decaffeination process. Altough many *Pseudomonas* spp. (*P. putida*, *P.* stutzeri, *P.*

Tabel 2. Various microorganisms used for the caffeine degradation

Microorganism	Caffeine concentration (g/l)	Incubation time (h)	Optimization approach (Yes[a]/No[b])	% Caffeine removal	References
►*Pseudomonas pseudoalcaligenes* TPS8	2.5	72	No	80.2	[Current study]
Pseudomonas stutzeri Gr21ZF	1.2	24 48	No Yes	59 86	[8]
Pseudomonas sp. S7	5	24	Yes	98.6	[31]
Pseudomonas putida CT25	2	72	Yes	50	[10]
Trichosporon asahii	2	96	Yes	100	[15]
Pseudomonas putida CBB5	2.5	192 20	No Yes	100 100	[35]
Pseudomonas sp. GSC 1182	1.2	48	No	80	[11]
Mix cultres of *Klebsiella/ Rhodococcus* sp.	0.5	10	Yes	100	[17]
Serratia marcescens	0.6	72	No	100	[18]
Pseudomonas putida	5	50	No	95	[34]
Stemphyllium sp.	0.19	29	Yes	100	[27]

[a]: Caffeine degradation experiments were performed under optimal conditions (addition of external carbon and nitrogen sources and/or optimize environmental culture conditions).

[b]: Caffeine degradation experiments were performed in the presence of caffeine as sole carbon and nitrogen source and caffeine removal yields were achieved without further optimization.

aeruginosa and *P. alcaligenes*) strains have been reported for the caffeine degradatiobn, no reports is available on caffeine removal using *P. pseudoalcaligenes.* Comparing the results obtained at this study with earlier ones encourages us to conclude that the growing cells of *P. Pseudoalcaligenes* TPS8 can be efficiently used as a cost- effective biocatalyst for the preparative treatment of the caffeine containing solutions. Further studies on this way to obtain higher yields of caffeine removal are in progress.

References

1. Batish D.R., Singh H.P., Kaur M., Kohli R.K., Yadav S.S. Caffeine affects adventitious rooting and causes biochemical changes in the hypocotyl cuttings of mung bean (*Phaseolus aureus* Roxb.). *Acta*. *Physiol*. *Plant*. **30**: 401-405 (2008).
2. Brand D., Pandey A., Roussos S., Soccol C.R. Biological detoxification of coffee husk by filamentous fungi using a solid state fermentation system. *Enz*. *Microb*. *Technol*. **27**: 127-133 (2000).
3. Buerge I.J., Poiger T., Muller M.D., Buser H.R. Caffeine, an anthropogenic marker for wastewater contamination of surface waters. *Environ*. *Sci*. *Technol*. **37**: 691-700 (2003).
4. Choi G.W., Um H.J., Kim Y., Kang H.W., Kim M., Chung B.W., Kim Y.H. Isolation and characterization of two soil derived yeasts for bioethanol production on Cassava starch. *Biomass*. *Bioenerg*. **34**: 1223-1231 (2010).
5. Cowan S.T., Steel K.J. Manual for the Identification of Medical Bacteria, 2nd Ed., University Press, London, pp. 94-150 (1993).
6. Dash S.S., Gummadi S.N. Catabolic pathways and biotechnological applications of microbial caffeine degradation. *Biotechnol*. *Lett*. **28**: 1993-2002 (2006).
7. Dash S.S., Gummadi S.N. Degradation kinetics of caffeine and related methylxanthines by induced cells of *Pseudomonas* sp. *Curr*. *Microbiol*. **55**: 56-60 (2007).
8. El-Mched F., Olama Z., Holail H. Optimization of the environmental and physiological factors affecting microbial caffeine degradation and its application in caffeinated products. *Basic*. *Res*. *J*. *Microbiol*. **1**: 17-27 (2013).
9. Erato M.S., Laura C., Ambra P., Guido M. Maternal caffeine consumption and sine causa recurrent miscarriage. *Eur*. *J*. *Obstet*. *Gynaecol*. *Reprod*. *Biol*. **158**: 220-224 (2011).
10. Fan F.Y., Xu Y., Liang Y.R., Zheng X.Q., Borthakur D., Lu J.L. Isolation and characterization of high caffeine-tolerant bacterium strains from the soil of tea garden. *Afr*. *J*. *Microbiol*. *Res*. **5**: 2278-2286 (2011).
11. Gokulakrishnan S., Chandraraj K., Gummadi S.N. A preliminary study of caffeine degradation by *Pseudomonas* sp. GSC1182. *Int*. *J*. *Food*. *Microbiol*. **113**: 346-350 (2007).
12. Gutiérrez-Sánchez G., Roussos S., Augur C. Effect of caffeine concentration on biomass production, caffeine degradation, and morphology of *Aspergillus tamarii*. *Folia*. *Microbiol*. **58**: 195-200 (2013).
13. Hallstrom T., Wolk A., Glynn A., Michaelsson K. Coffee, tea and caffeine consumption in relation to osteoporotic fracture risk in a cohort of Swedish women. *Osteoporos*. *Int*. **17**: 1055-1064 (2006).
14. Heckman M.A., Weil J., De Mejia E.G. Caffeine (1, 3, 7-trimethylxanthine) in foods: A comprehensive review on consumption, functionality, safety, and regulatory matters. *J*. *Food*. *Sci*. **75**: 77-87 (2010).
15. Lakshmi V., Das N. Caffeine degradation by yeasts isolated from caffeine contaminated samples. *Int*. *J*. *Sci*. *Nat*. **1**: 47-52 (2010).

16. Löffler F.E., Sun Q., Li J., Tiedje J.M. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. *Appl*. *Microbiol*. *Biotechnol*. **66**: 1369-1374 (2000).
17. Madyastha K.M., Sridhar G.R. A novel pathway for the metabolism of caffeine by a mixed culture consortium. *Biochem*. *Biophys*. *Res*. *Commun*. **249**: 178-181 (1998).
18. Mazzafera P., Olsson O., Sandberg G. Degradation of caffeine and related methylxanthines by *Serratia marcescens* isolated from soil under coffee cultivation. *Microb*. *Ecol*. **31**: 199-207 (1996).
19. Mohapatra B.R., Harris N., Nordin R., Mazumdar A. Purification and characterization of a novel caffeine oxidase from *Alcaligenes* species. *J. Biotechnol*. **125**: 319-327 (2006).
20. Nayak S., Harshitha M.J., Sampath M.C., Anilkumar H.S., Rao C.V. Isolation and characterization of caffeine degrading bacteria from coffee pulp. *Indian*. *J. Biotechnol.* **11**: 86-91 (2012).
21. Pai P.V., Pai A., Pai S., Devadiga S.Y., Nayak V., Rao C.V. Effect of glucose and nitrogen source on caffeine degradation by four filamentous fungi. *Indian. J. Biotechnol*. **12**: 432-434 (2013).
22. Park H.S., Im N.G., Kim K.H. Extraction behaviors of caffeine and chlorophylls in supercritical decaffeination of green tea leaves. *LWT – Food. Sci. Technol*. **45**: 73-78 (2012).
23. Roussos S., Hannibal L., Aquiahuatl M.A., Hernandez M.R.T., Marakis S. Caffeine degradation by *Penicillium verrucosum* in solid-state fermentation of coffee pulp—critical effect of additional inorganic and organic nitrogen sources. *J*. *Food*. *Sci*. *Tech*. *Mys*. **31**: 316-319 (1994).
24. Sambrook J., Fritsch E.F., Maniatis T. Molecular cloning: a laboratory manual, 2nd Ed., Cold Springs Harbor Laboratory, Cold Spring Harbor, N.Y (1989).
25. Sarath Babu V.R., Patra S., Thakur M.S., Karanth N.G., Varadaraj M.C. Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708. *Enzyme. Microb. Technol.* **37**: 617-624 (2005).
26. Sarath Babua V.R., Patraa S., Karantha N.G., Kumarb M.A., Thakur M.S. Development of a biosensor for caffeine. *Anal. Chim. Acta*. **582**: 329-334 (2007).
27. Schwimmer S., Kurtzman R.H. Caffeine metabolism by *Penicillium roqueforti*. *Arch*. *Biochem*. *Biophys*. **147**: 109-113 (1971).
28. Snel J., Lorist M.M. Effects of caffeine on sleep and cognition. *Prog. Brain*. *Res*. **190**: 105-117 (2011).
29. Srivastava K.C. Properties of thermostable hemicellulolytic enzymes from *Thermomonospora* strain 29 grown in solid state fermentation on coffee processing solid waste. *Biotechnol. Adv*. **11**: 441-65 (1993).
30. Swati S.D., Sathyanarayana N.G. Inhibitory effect of caffeine on growth of various bacterial strains. *Res. J. Microbiol*. **3**: 457-465 (2008).
31. Syed Baker., Sahana S., Rakshith D., Kavitha H.U., Kavitha K.S., Satish S. Biodecaffeination by endophytic *Pseudomonas* sp. isolated from *Coffee arabica* L. *J. Pharm. Res*. **5**: 3654-3657 (2012).
32. Tamura K., Dudley J., NeiM., Kumar S. Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol*. *Biol*. *Evol*. **24**: 1596–1599 (2007).
33. Washington J.A., Sutter V.L. *Dilution susceptibility test: agar and macro-broth dilution procedures*. In: Lennette EH, Balows A, Hausler JR, WJTruant, J. (Eds.). *Manual of Clinical Microbiology*, 3nd Ed., American Society for Microbiology, Washington DC, pp. 453-458 (1980).
34. Woolfolk C.A. Metabolism of *N*-methylpurines by a *Pseudomonas putida* strain isolated by enrichment on caffeine as the sole source of carbon and nitrogen. *J. Bacteriol*. **123**: 1088-1106 (1975).
35. Yu C.L., Louie T.M., Summers R., Kale Y., Gopishetty S., Subramanian M. Two distinct pathways for metabolism of theophylline and caffeine are coexpressed in *Pseudomonas putida* CBB5. *J. Bacteriol*. **191**: 4624-4632 (2009).