Progress in Biological Sciences
Vol. 3, Number 2, Summer/Fall 2013/145-156

Saccharomyces cerevisiae TFS9, a novel isolated yeast capable of high caffeine-tolerant and its application in biodecaffeination approach

Received: September 13, 2013; Accepted: July 10, 2013

Morahem Ashengroph[1]*; Maryam Borchaluei[2]

1- Assistant Professor of Microbiology, Department of Biology and Biotechnology, Faculty of Sciences, University of Kurdistan 2- MSc. Student of Molecular Cell Biology, Department of Biology and Biotechnology, Faculty of Sciences, University of Kurdistan

Abstract

There is a great call for using microbial bio-decaffeination approach to remove caffeine from caffeinated products and industrial wastes. We aimed in this study to screen strains of yeasts which exhibit high caffeine tolerance and to investigate the bio-degradation of caffeine under growth conditions. Sixteen yeast strains were isolated from the cultivated tea soils collected from sites of northern Iran and evaluated for the caffeine tolerance by the agar dilution method. Based on the tolerance efficiency, strain TFS9 was selected and identified as *Saccharomyces cerevisiae* TFS9 (GenBank accession number **KF414526**) on the morphological and bioochemical characteristics as well as molecular phylogenetic studies based on amplification the ITS1–5.8S–ITS2 rDNA sequences. The time course of caffeine removal by growing cells of the strain TFS9 in the minimal salt medium containing caffeine as the sole source of carbon was estimated by a decrease in caffeine absorbance using UV-visible spectrophotometer. The concentration of caffeine in the supernatant of the yeast culture medium decreased by 84.8% (from 3.5g/l to 0.53 g/l) after 60h of incubation by using of *S. cerevisiae* TFS9, without additional optimization process. Results of experimental studies suggest a simple and cost-effective process for the microbial decaffeination of caffeine-containing solutions, and provide a promising approach for developing safe processes that can be used effectively for decaffeination of industrial effluents. The present study provides the first evidence on the caffeine bio-degradation using yeast species of *S. cerevisiae*.

Key Words: bio-decaffeination, caffeine, *saccharomyces cerevisiae* TFS9, tolerance pattern.

* **Corresponding author:** m.ashengroph@uok.ac.ir

PROGRESS IN BIOLOGICAL SCIENCES

Introduction

Caffeine (1, 3, 7-trimethylxanthine) is a purine alkaloid which has been added with significant levels in most popular beverages, particularly coffee, tea, caffeinated cola and soda (1). Caffeine is also present in large quantities in food products such as chocolate, desserts and pastries (1, 2). In spite of such distribution in beverages and food preparations, higher level of caffeine consumption through beverages and confectionary products led to higher risk of human health problems like headache, fatigue, abnormal muscle activity, heart irregularity, adrenal simulation, abnormal renal function and bone problems such as osteoporosis and alveolar bone loss in ligature-induced periodontitis (3, 4). Ingestion of higher level of caffeine during pregnancy can increase the risk of fetal deformity and restrict fetal growth (5).

Considering the adverse effects of caffeine in widely consumed beverages like caffee and tea, caffeine free products are desirable (6). Caffeine is also one of the major agro-industrial wastes generated in large quantities in tea and coffee cherry processing plants, which are often released into surface waters, making a major source of the pollution of rivers, lakes and environment in the vicinity of the coffee processing sites (7). The presence of caffeine in the soil also affects soil fertility as it inhibits seed germination and growth of seedlings (8). Consequently, elimination of caffeine is very important from an environmental standpoint. Conventional methods like solvent extraction and use of supercritical fluid extraction are being used to remove toxic caffeine. However, these approaches are presently not favored because of the toxicity of some of the reagents, the high cost of organic solvents, and theat the procedures are time consuming (9).
Vhe conventional methods are non-specific for elimination of caffeinated products and sometimes remove flavor elements in the products. Therefore, their use is accompanied with incomplete removal of caffeine and production of unpalatable products (10).

Given the concerns outlined above, developing environmentally-friendly processes and specific decaffeination approaches such as microbial decaffeination is desirable. Caffeine is toxic to many microorganisms; however, some microorganisms are able to grow in the presence of caffeine and to degrade thealkaloid (11, 12).

The first microbial degradation of caffeine was reported in strains of *penicillium roqueforti* and Stemphylum sp. (13). To date, large number of *bacteria* and molds strains belonging to *Pseudomonas* sp. (10, 14-16), *Serratia* sp. (17), *Rhizopus* sp. (12), *Bacillus* sp. (18, 19), *Aspergillus* sp. (20), *Klebsiella* and *Rhodococcus* sp. (21) have been reported to be capable of caffeine degradation. There are, however, a few reports regarding caffeine degradation associated with yeast strains. Lakshmi and Das (22) reported a novel isolated yeast strain belonging to *Trichosporon asahii* based on the caffeine degradation efficiency. In the current study, isolation and characterization of *Saccharomyces cerevisiae* TFS9 with its capability of high caffeine-tolerant followed by caffeine degradation experiments are reported. To the best of our knowledge, the present research is the first study investigating the degradation of caffeine using growing cells of *S. cerevisiae*.

Materials and methods

Chemicals and culture media

Pure caffeine (>99%) used for bio-decaffeination experiments was obtained

from Sigma (St. Louis, Missouri, USA). Stock solutions of caffeine were prepared in distilled water, protected from light and stored at 4º C following filtration using sterile syringe filter units (0.22 μm pore size). Dichloran Rose-Bengal Chloramphenicol Agar was purchased from Quelab, UK. Glucose was acquired from Merck (E. Merck, Darmstadt, Germany). The Agar was prepared from Difco (Detroit, MI, USA). Bacteriological peptone and yeast extract were obtained from Quelab, UK. Yeast Nitrogen Base (YNB) media have been prepared from Himedia, India. All other chemicals used were commercially available and of analytical grade.

Sample collection and screening of yeasts

Sixteen soil samples were collected from cultivated tea fields in Northern Iran and used to screen novel yeast strains capable of high tolerance caffeine concentrations under an enrichment culture. One gram of collected soil sample was added to 100 ml of sterilized distilled water; serial dilution was prepared and spread on a Dichloran Rose-Bengal Chloramphenicol Agar containing (g/l): peptone, 5.0; glucose, 10.0; potassium dihydrogen phosphate, 1.0; magnesium sulphate, 0.5; dichloran, 0.002; rose-bengal, 0.025; chloramphenicol, 0.1; agar, 15 and supplemented with 1.0 g/l of caffeine and then incubated aerobically in a shaker with agitation speed of 150 rpm at 28º C for 5 days. Single yeast colonies were picked, purified and maintained on yeast peptone dextrose (YPD) (1% yeast extract, 2% peptone, 2% glucose, 2% agar pH 5.8 ±0.1) slant medium containing 1 g/l of caffeine. The successful cultures were selected to determine their caffeine tolerance pattern using the agar dilution method of Washington and Sutter (23).

Determination of caffeine tolerance

For the caffeine tolerance experiment, 20 ml of melted YPD and modified M9 defined minimal salt from Sambrook et al. (24) [glucose 5g/l; NH_4Cl 2.5g/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 100 mM pH 5.6] agar media supplemented with different concentrations of caffeine ranging from 2.5 to 20 g/l were prepared and poured into plates of 8 cm in diameter. Ten microliter of 1 McFarland standard of yeast suspension (3×10^8 CFU/ml), prepared from yeast 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 yeast strains was determined visually. Each plate was run in triplicates.

The study of cell growth and caffeine degradation under growing cells of strain TFS9

A loop full of actively grown culture of high caffeine-tolerant strain TFS9 on YPD agar fortified with 1 g/l caffeine was transferred to 250-ml flask containing 50 ml of mineral M9 medium ($MgSO_4.7H_2O$ 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 100 mM pH 5.6± 0.1) supplemented with different concentrations of caffeine (0.5-5 g/l) as the sole carbon and nitrogen source at 28º C on a rotary shaker (15 rpm) for 84 h. Control was prepared without inoculating yeast strain under identical conditions. Time-course samples were withdrawn at different time intervals and subjected to cell growth analysis and residual caffeine in a UV–Vis spectrophotometer (Specord 210, Carel Zeiss Technology, Germany). The culture growth was monitored 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 (25). The elimination of caffeine in the culture media was recorded by decrease in absorbance at 278 nm in a UV- visible Analytik Jena's spectrophotometer SPECORD 210 (22). The cells were centrifuged at 8000 rpm for 10 min at 4º C and the supernatants were used to determine 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 (16). Calibrating caffeine solutions were prepared by diluting standard solutions to a concentration range of 5 to 35 mg/l. Good linearity for the concentration intervals examined as exhibited by the equation and the coefficient of determination for the calibration plot $y = (0.0424)x + 0.0263$ and $R^2 = 0.9973$. All experiments were carried out in triplicate and the means of three separate experiments with the standard deviation shown by vertical bars.

Phenotypic Characterization and phylogenetic analysis of the selected yeast strain TFS9

The morphological and physiochemical characterization of the yeast strain TFS9 was carried out by using the methods proposed by Kurtzman and Fell (26). The tests included colony color, shape and texture, carbon and nitrogen assimilation tests which were examined in minimal media YNB and YCB supplemented with different carbon and nitrogen sources and incubated at 25º C for 1 to 3 weeks. The Urea hydrolysis was tested on Urea R broth (Difco). Genomic DNA of the strain was extracted using the method of glass bead disruption (27). The universal primers for amplification of the ITS1–5.8S–ITS2 rDNA regions in the yeast were used, the forward primer ITS1 (5′-tccgtaggtgaacctgcggg-3′) and reverse primer ITS4 (5′-tcctccgcttattgatatgc-3′) (28). PCR reactions were conducted in a volume of 25µl containing 19.2 µl of PCR water (Fermentase), 0.1µl Dream TaqTM DNA Polymerase (5U/ µl, Fermentase), 2.5 µl of 10 X PCR buffer (20 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, stabilizing agent and 50% (v/v) glycerol, Fermentase), 2 µl dNTPs (2.5 mM, Fermentase), 0.1µl of each primer (125µM, Primm srl) and 1µl DNA template (1µg/ µl). Amplification was performed in a PCR system thermocycler programmed which was set to initial denaturation at 95°C for 2 min, followed by 32 cycles of 95º C for 45 s, annealing at 55º C for 45 s, and extension at 72º C for 2 min. This was followed by a final elongation step for 10 min at 72º C. After amplification, the PCR products from strain TFS9 were analyzed using 1.5% agarose gel electrophoresis and purified by QIAquick Gel Extraction (QIAGEN). The purified PCR product was sequenced in both directions using an automated sequencer by Macrogen Company (Seoul, Korea) and was analyzed using the Basic Local Alignment Search Tool (BLAST) search program (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic tree was constructed based on Kimura's two-parameter and Neighbor-Joining method by using the MEGA version 4 software package (29).

Results

Isolation of high caffeine-tolerant yeasts

Sixteen colony yeasts were screened from cultures in Dichloran rose-bengal chloramphenicol agar media supplemented with 1 g/l of caffeine and

were designated as isolates TFS1-TFS16. In order to achieve efficient bio-decaffeination, it is necessary to apply an efficient yeast strain that can tolerate high caffeine concentrations. As such, caffeine was added to to YPD agar and synthetic defined minimal salt media at concentrations ranging from 2.5 to 20 g/l and the intrinsic tolerance of the yeast strains to caffeine was evaluated (Fig. 1). The results suggested that the isolate TFS9 has maximum caffeine tolerances, with a tolerance of 12.5 and 20 g/l in the synthetic defined and complex media, respectively (Fig. 1). Due to its maximum tolerance, strain TPS8 was selected, characterized and examined for bio-decaffeination experiments.

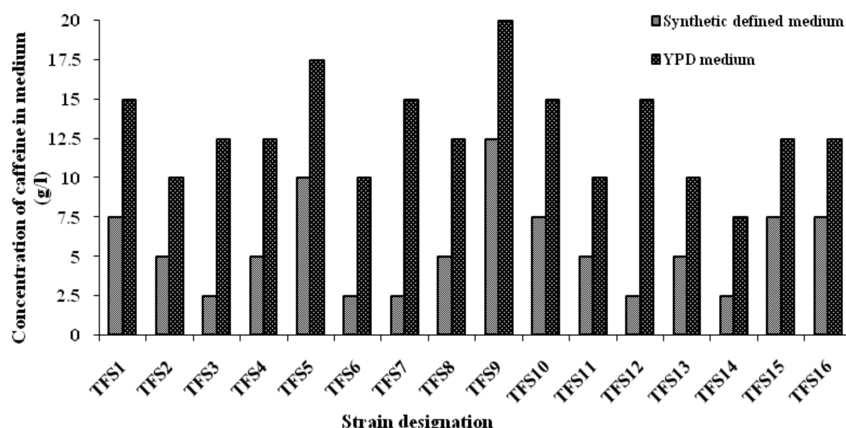

Figure 1. Caffeine tolerance capacity by isolated yeast strains.

Morphological and Molecular characterization of isolated yeast TFS9

Characterization of the TFS9 yeast strain was based on morphological and biochemical tests as well as rDNA sequencing and molecular phylogenetic analysis. On YPD agar medium, the streak culture of the colony of the isolate TFS9 had a cream-white and smooth surface after 24 h at 28º C (Fig. 2B and C). Microscopically, the isolate TFS9 formed yeast-like colonies that reproduced by budding. Pseudohyphae and true hyphae were not formed. The colonies were observed as singles, pairs or in groups (Fig. 2A).

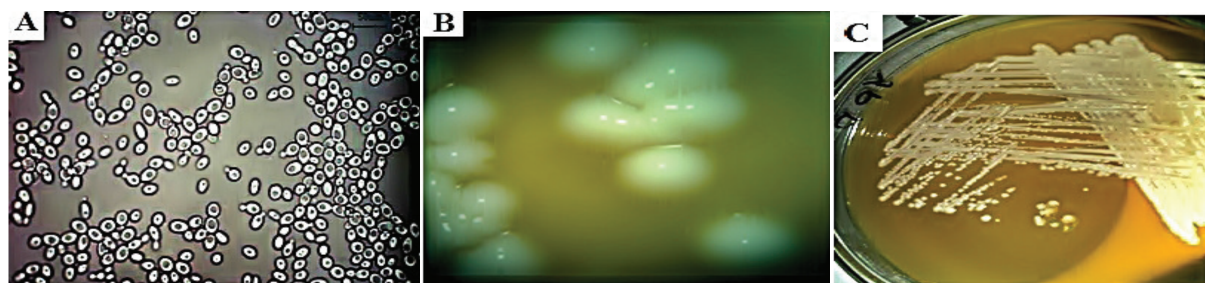

Figure 2. Morphological and cultural analysis of isolated yeast strain *S. cerevisiae* TFS9. (A) Light microscopy micrograph showing the cell morphology of strain TFS9 when grown in YPD broth for 24h. (B) Stereo microscope of the colony of yeast strain TFS9 when grown in YPD agar for 48h. (C) The culture color became apparent in the cultures of the yeast strain TFS9 when grown in YPD agar within 48h.

The carbon and nitrogen sources assimilation tests of isolate TFS9 were then studied. Table 1 illustrates the results obtained on the taxonomic identification of the isolate TFS9. Based on the morphological and nutrient assimilation properties (Table 1), we found that the isolated yeast strain TFS9 was closely related to *Saccharomyces cerevisiae*.

Table 1. Taxonomic characterization caffeine-degrading yeast strain TFS9.

Characteristics	Strain TFS9
Shape	round to short oval
Arrangement	singly, in pairs and groups
Color on Agar	white
Surface on Agar	shiny
Texture on agar	smooth
Cell division	budding
Pseudomycelium	absent
True Mycelium	absent
Nutrient source assimilation:	
Glucose	positive
Galactose	negative
Sucrose	positive
Maltose	positive
Raffinose	positive
Lactose	negative
Xylose	negative
Manitol	negative
Cellobiose	negative
Glucoseamine	positive
KNO_3	negative
Ethylamine	negative
$(NH4)_2SO_4$	positive
Lysine	negative
Urease activity	negative
Starch hydrolysis	negative

To confirm its phylogenetic relationship with *S. cerevisiae*, genomic DNA was extracted and gene coding for ITS1-5.8S-ITS2 rDNA regions was amplified as described in materials and methods section. The obtained amplified PCR product was about 840 bp in length (Fig. 3).

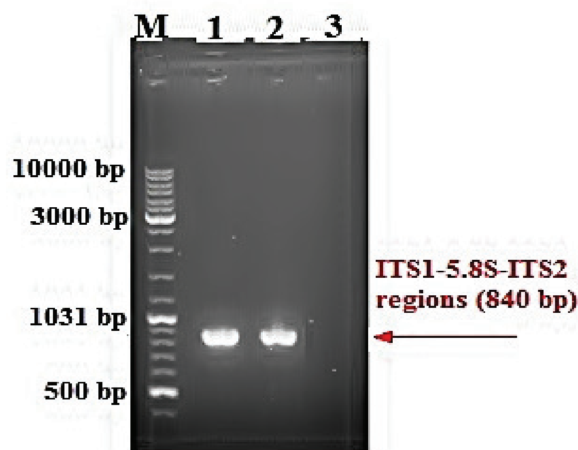

Figure 3. PCR products of rDNA of strain TFS9. Lane M: Molecular weight markers (GeneRulerTM DNA ladder Mix); Lane 1: *S. cerevisiae* strain TFS9; Lane 2: *S. cerevisiae* PTCC5052 (Positive control); Lane 3: Negative control (no added DNA).

ITS1-5.8S-ITS2 rDNA sequences of isolate TFS9 were submitted to the National Center for Biotechnology Information (NCBI) Databases under accession number **KF414526**. Comparative analysis of the partial rDNA sequences of isolate TFS9 with available sequence databases showed that partial ITS1-5.8S-ITS2 rDNA sequences of the isolate is over than 99% homology to *S. cerevisiae* (GeneBank accession no KC544501). In order to determine the relationship between isolate TFS9 and other species of the genus *S. cervisiaea* shared highest sequence similarities, phylogenetic tree based on the partial ITS1-5.8S-ITS2 rDNA sequences was constructed (Fig. 4). Therefore, novel isolated yeast strain TFS9, capable of caffeine degradation, was identified as *Saccharomyces cerevisiae* TFS9 based on the morphological, physiochemical and molecular data.

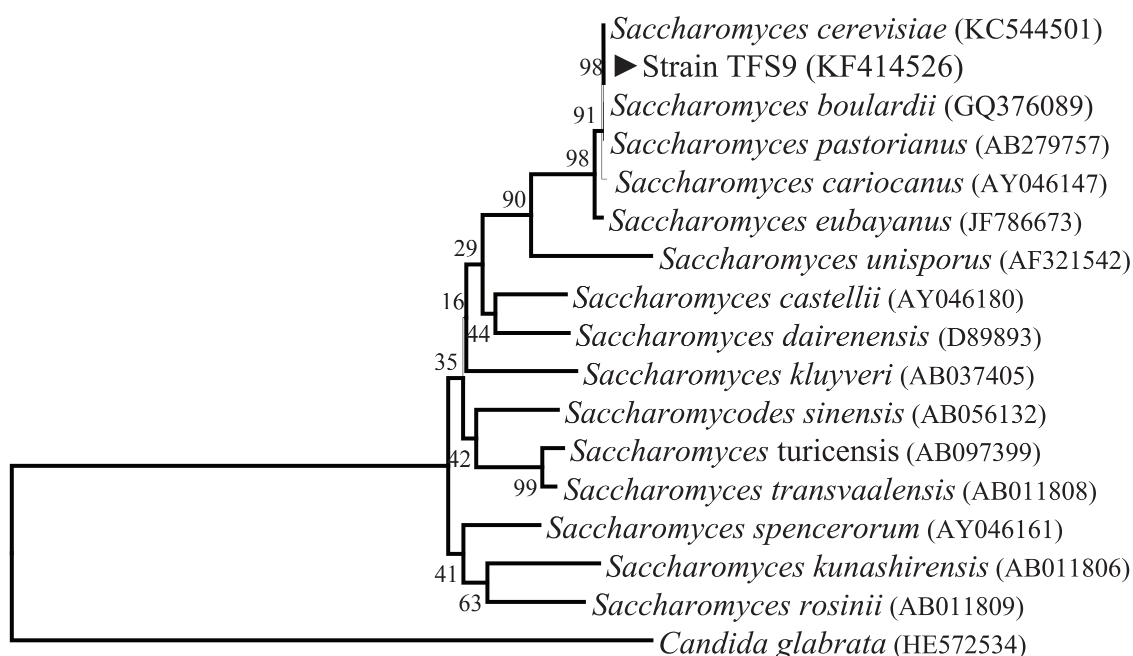

Figure 4. Phylogenetic relationship of yeast strain TFS9 and the related taxa based on amplification of ITS1–5.8S–ITS2 rDNA regions. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. *Candida glabrata* was used as outgroup. Scale bar represents 0.02 substitutions per nucleotide position. GenBank accession numbers are given in parentheses.

Kinetic study of cell growth and caffeine consumption by *S. cervisiea* TFS9

To study the effect of caffeine concentration on the cell density and caffeine degradation by *S. cervisiaea* strain TFS9, the basal minimal media (as mentioned under Materials and Methods) were supplemented with caffeine ranging from 0.5 to 5 g/l (Fig. 5A, B). Growth curve of the *S. cervisiaea* TFS9 at different concentration of caffeine is shown in Figure 5A. It is clear from the result that the amount of initial concentration of caffeine in the medium has a significant effect on the cell growth. As shown by the determination of the biomass levels at different caffeine supplementation (Fig. 5A) , increasing the concentration of caffeine from 0.5 to 3.5 g/l increased the cell growth by the strain during bio-decaffeination process and the highest dry weight (0.42 g/l) is obtained at this concentration after 60 h incubation. The results showed that the supplementation of caffeine especially at higher concentration has a deleterious effect on cell density during the caffeine degradation with *S. cervisiea* TFS9 under growing cell conditions. Simultaneously, different concentrations of caffeine were provided in the basal minimal media to study their effect on the removal of caffeine using growing cells of *S. cervisiea* TFS9. As shown in Figure 5B, increase in the initial concentration of caffeine from 0.5 up to 3.5 g/l supported high caffeine degradation and around 84.8% of initial caffeine (from 3.5 g/l to 0.53 g/l) was degraded by strain TFS9 within the 60 h of incubation. In contrast, caffeine at concentrations higher than 3.5 g/l resulted in lower degradation, due to the inhibitory effect of caffeine on the growth of the organism (Fig. 5B).

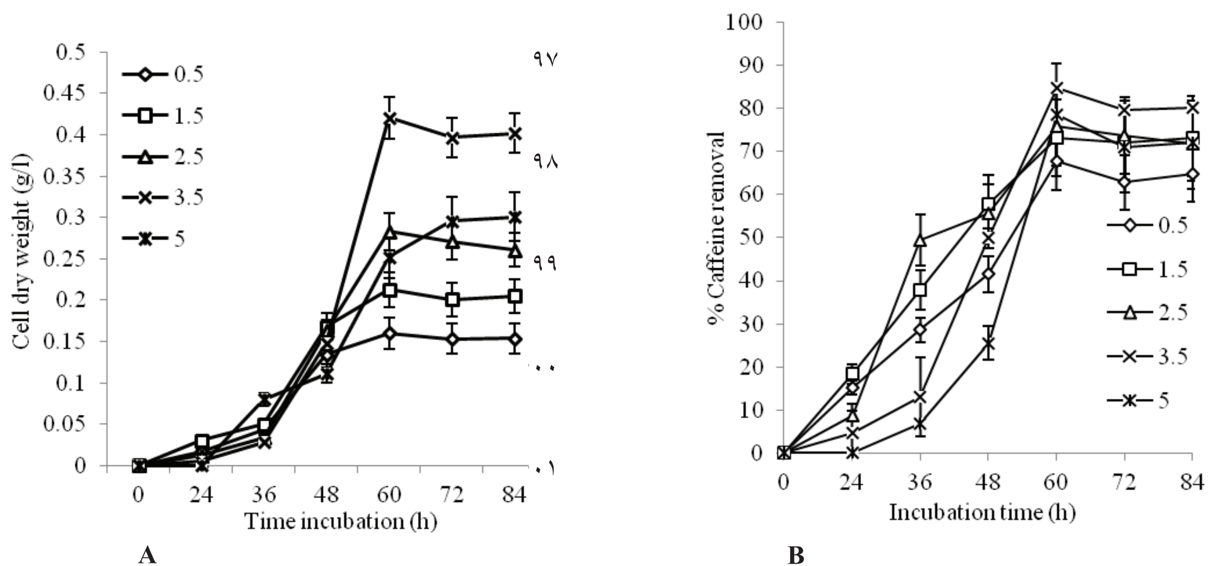

Figure 5. Time course of degradation of caffeine by growing cells of *Saccharomyces cerevisiae* TFS9. (A) Growth Curve of yeast strain TFS9 in the MM9 medium fortified with different caffeine concentrations. (B) Effect of initial caffeine concentration on its degradation by isolate TFS9 in the MM9 medium. Results represent the means of three separate experiments, and deviation bars indicated.

Discussion and Conclusion

Caffeine and other xanthine derivatives have been extensively used in various industries, including food, beverages, pharmaceutical and agronomy industries (30, 31). The presence of toxic caffeine in effluents has increased concerns over water and soil contamination. In the recent years, a growing interest has been directed toward the possible role of microorganisms in caffeine detoxification approach. Many types of physiochemical methods like solvent extraction with various toxic solvents such as chloroform, ethyl acetate and dichloromethane, water diffusion and also supercritical CO_2 extraction have been used to decrease the toxicity of caffeine in aqueous solutions. However, these methods impose limitations like adverse effects because of solvent residues, loss of essential flavors and high operational costs, and hence are not considered suitable detoxification approaches (32, 33). To overcome these limitations, recently, a green method using either microbial cells or enzymes has been proposed for commercial decaffeination approach. A number of researchers have shown that selective elimination of caffeine in caffeine -containing solutions by caffeine degrading microorganisms. In bacteria, *Pseudomonas* species and among fungi *Aspergillus* and *Penicillium* species are efficient in degradation of caffeine (11, 20). Due to the toxicity of caffeine to microorganisms, isolation and characterization of strains having a high tolerance to caffeine would enable us to screen efficient species for bio-degradation of toxic caffeine. In the present study, we isolated 16 yeast strains from tea fields in Iran by an enrichment procedure (designated as TFS1-TFS16) and evaluated their tolerance pattern to caffeine (Fig. 1). Among these, strain TFS9 was shown to have the maximum caffeine tolerance ability and a potential to degrade this compound under ambient condition. The strain was identified as *Saccharomyces cerevisiae*

TFS9 based on the results of morphological characteristics, physiological and biochemical characteristics as well as ITS1-5.8S-ITS2 rDNA regions analysis. Previously, a few yeast strains such as *Trichosporon* sp., *Candida* sp. and *Kloeckera* sp. have been explored for the degradation of caffeine (22). However, to the best of our knowledge, this study brings the first report for the caffeine degradation by *Saccharomyces cerevisiae*. Growing cells of *S. cerevisiae* TFS9 were also tested for their ability to degrade caffeine under different caffeine concentrations. Figure 5 illustrates the results of effects of initial caffeine concentrations and degradation time on cell growth and percentage of caffeine degradation with *S. cerevisiae* TFS9 . As shown in Figure 5A, the cell density increased as the amount of caffeine increased to 3.5 g/l. However, higher amounts of caffeine decreased the cell growth, probably as a result of caffeine inhibition. As shown in Figure 5B, the maximum caffeine removal was observed after 60 h using 3.5 g/l of caffeine. Based on these results, using 3.5 g/l caffeine, the maximal growth cell (cell dry weight of 0.42 g/l) and highest caffeine degradation (84.8%) by *S. cerevisiae* TFS9 was achieved after 60 h under growing cell conditions. It should be stressed that, these yields which were obtained in a non-optimized process, confirm the considerable potential of growing cells of *S. cerevisiae* TFS9 as a biocatalyst for removal of caffeine from caffeine containing solutions. The first microbial bio-degradation of caffeine was reported using strains of *Penicillium roqueforti* and *Stemphylum* sp. (13). However, the strains were capable to degrade caffeine after a 29-h reaction using 0.19 g/l of caffeine under optimized conditions. Mazzafera *et al*. (17) 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. A strain of *Pseudomonas stutzeri* Gr21ZF has been reported for caffeine degradation which is able to degrade 59% of caffeine at concentration 1.2 g/l after 24 h (10). Woolfolk (34) reported the caffeine degradation with strain of *Pseudomonas putida* which showed 95% degradation in 95 h when caffeine with initial concentration of 5g/l was used as the sole carbon and nitrogen source. The bio-degradation of caffeine was also reported with strains of *Pseudomonas stutzeri*. With *Pseudomonas stutzeri* Gr21ZF, 59.0% of caffeine was removed at 1.2 g/l initial caffeine concentration after 24 h (10). Attempts have been made to improve the caffeine removal yield using optimization process by varying the environmental parameters (pH, temperature, shaking speed) and nutrient condition (additional of external carbon and nitrogen sources). Madyastha and Sridhar (21) developed an optimization processe for caffeine degradation with *Klebsiella* and *Rhodococcus* sp. by adding glucose and reported 100% caffeine degradation after 10 h of incubation with initial caffeine concentration of 0.5 g/l. Hakil *et al*. (20) reported 67.2% caffeine removal (initial caffeine concentration of 1.2 g/l) after 48 h incubation by a strain of *Aspergillus tamari*. In a study of caffeine degradation, a novel yeast strain of *Trichosporon asahii* was reported which showed 100% caffeine degradation within 96 h hours in the presence of 5 g/l of sucrose under optimized culture conditions (22). Compared with the previous studies, our results showed that using growing cells of *S. cerevisiae* TFS9, without the addition of external carbon and nitrogen sources or other further optimization protocols allowed reasonable caffeine degradation. . In conclusion, we have shown that *S. cerevisiae*

TFS9, a novel isolated strain from cultivated tea fields, which has high ability for caffeine tolerance (up to 20 g/l) and caffeine degradation (84.8 %), can be used to remove toxic caffeine from agro-industrial waste. Further research is needed to improve this approach and to scale up the caffeine degradation up to the desired level in progress.

Acknowledgement

This study was supported by a grant from the Postgraduate Administration Office of the University of Kurdistan to M. bercheloii for obtaining a MSc. Degree.

References

1. Weigel, U., Berger, E., Jensen, R., Kallenborn, H. and Thoresen, H. (2004) Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromso/Norway with emphasis on ibuprofen and its metabolites. *Chemosphere*., 56, 583-592.
2. Anaya, A.L., Cruz-Ortega, R. and Waller, G.R. (2006) Metabolism and ecology of purine alkaloids. *Front. Biosci*., 11, 2354-2370.
3. Hallstrom, T., Wolk, A., Glynn, A. and Michaelsson, K. (2006) Coffee, tea and caffeine consumption in relation to osteoporotic fracture risk in a cohort of Swedish women. *Osteoporos*. *Int*., 17, 1055–1064.
4. Bezerra, J.P., da Silva, L.R., de Alvarenga Lemos, V.A., Duarte, P.M. and Bastos, M.F. (2008) Administration of high doses of caffeine increase alveolar bone loss in ligature-induced periodontitis in rats. *J. Periodontol*., 79, 2356-2360.
5. CARE Study Group (2008) Maternal caffeine intake during pregnancy and risk of fetal growth restriction: a large prospective observational study. *BMJ*., 337, a2332.
6. Greenberg, J.A., Dunbar, C.C., Schnoll, R., Kokolis, R., Kokolis, S. and Kassotis, J. (2007) Caffeinated beverage intake and the risk of heart disease mortality in the elderly: a prospective analysis. *J. Clin*. *Nutr*., 85, 392–398.
7. Buerge, I.J., Poiger, T., Muller, M.D. and Buser, H.R. (2003) Caffeine, an anthropogenic marker for wastewater contamination of surface waters. *Environ*. *Sci*. *Technol*., 37, 691-700.
8. Batish, D.R., Singh, H.P., Kaur, M., Kohli, R.K. and Yadav, S.S. (2008) Caffeine affects adventitious rooting and causes biochemical changes in the hypocotyl cuttings of mung bean (*Phaseolus aureus Roxb*.). *Acta*. *Physiol*. *Plant*., 30, 401-405.
9. Yadav, S.K. and Ahuja, P.S. (2007) Towards generating caffeine-free tea by metabolic engineering. *Plant*. *Foods*. *Hum*. *Nutr*., 62, 185-191.
10. El-Mched, F., Olama, Z. and Holail, H. (2013) Optimization of the environmental and physiological factors affecting microbial caffeine degradation and its application in caffeinated products. *Basic*. *Res*. *J*. *Mcrobiol*., 1, 17-27.
11. Asano, Y., Komeda, T. and Yamada, H. (1993) Microbial production of theobromine from caffeine. *.Biosci. Biotechnol Biochem*., 57, 1286–1289.
12. Tagliari, C.V., Sanson, R.K., Zanette, A., Franco, T.T. and Soccol, C.R. (2003) Caffeine degradation by *Rhizopus* delemar in packed bed column bioreactor using coffee husk as substrate. *Braz*0*J. Microbiol*., 34, 102–104.
13. Schwimmer, S. and Kurtzman, R.H. (1971) Caffeine metabolism by *Penicillium* roqueforti. *Arch*. *Biochem*. *Biophys*., 147, 109-113.
14. Dash, S.S. and Gummadi, S.N. (2007) Degradation kinetics of caffeine and related methylxanthines by induced cells of Pseudomonas sp. *Curr*. *Microbiol*., 55, 56-60.
15. Fan, F.Y., Xu, Y., Liang, Y.R., Zheng, X.Q., Borthakur, D. and Lu, J.L. (2011) Isolation and characterization of high caffeine-tolerant bacterium strains from the soil of tea garden. *Afr*. *J*. *Microbiol*. *Res*., 5, 2278-2286.
16. Baker, S., Sahana, S., Rakshith, D., Kavitha, H.U., Kavitha, K.S. and Satish, S. (2012) Biodecaffeination by *.endophytic Pseudomonas sp* isolated from Coffee arabica L. *J. Pharm*. *Res*., 5, 3654-3657.
17. Mazzafera, P., Olsson, O. and Sandberg, G. (1996) Degradation of caffeine and related methylxanthines by *Serratia marcescens* isolated from soil under coffee cultivation. *Microbial*. *Ecology*., 31, 199-207.

18. Kurtzman, R.H. and Schwimmer, S. (1971) Caffeine removal from growth media by microorganisms. *Experimencia*., 127, 481- 482.

19. Ramarethinam, S. and Rajalakshmi, N. (2004) Caffeine in tea plants [Camellia sinensis (L.) O. Kuntze]: in situ lowering by *Bacillus licheniformis* (Weigmann) Chester. *Indian*. *J*. *Exp*. *Biol*., 42, 575–80.

20. Hakil, M., Denis, S., Viniegra-Gonzalez, G. and Augur, C. (1998) Degradation and product analysis of caffeine and related dimethylxanthines by filamentous fungi. *Enz. Microb*. *Technol*., 22, 355-359.

21. Madyastha, K.M. and Sridhar, G.R. (1998) A novel pathway for the metabolism of caffeine by a mixed culture consortium. *Biochem. Biophys*. *Res*. *Commun*., 249, 178-181.

22. Lakshmi, V. and Das, N. (2010) Caffeine degradation by yeasts isolated from caffeine contaminated samples. *Int. J*. *Sci*. *Nat*., 1, 47-52.

23. Washington, J.A. and Sutter, V.L. (1980) The dilution susceptibility test: agar and macro-broth dilution procedures. In: Manual of clinical microbiology, 3rd ed, Lennette EH, Balows A, Hausler WJ Jr, Truant JP eds. American Society for Microbiology; Washington DC, USA, pp. 453-458.

24. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Springs Harbor Laboratory, Cold Spring Harbor, NY.

25. Choi, G.W., Um, H.J., Kim, Y., Kang, H.W., Kim, M., Chung, B.W. and Kim, Y.H. (2010) Isolation and characterization of two soil derived yeasts for bioethanol production on Cassava starch. *Biomass*. *Bioenerg*., 34, 1223-1231.

26. Kurtzman, C.P. and Fell, J.W. (2000) The yeasts: a taxonomic study (4th revised and enlarged edition). Elsevier, Amsterdam, pp. 1–525.

27. Yamada, Y., Makimura, K., Mirhendi, H., Ueda, K., Nishiyama, Y., Yamaguchi, H. and Osumi, M. (2002) Comparison of different methods for extraction of mitochondrial DNA from human pathogenic yeasts. *Jpn. J*. *Infect*. *Dis*., 55, 122–125.

28. White, T.J., Bruns, T., Lee, S. and Taylor, J.T. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, New York, pp. 315-322.

29. Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*., 24, 1596–1599.

30. Barone, J.J. and Roberts ,H.R. (1996) Caffeine consumption. *Food. Chem. Toxicol*., 34, 119-129.

31. Juliano, L.M. and Griffiths, R.R. (2005) Caffeine. In Substance Abuse: A Comprehensive Textbook, 4th ed., Edited by J. H. Lowinson, P. Ruiz & R. B. Millman. Lippincott Williams & Wilkins, Philadelphia, PA.

32. Udayasankar, K., Manohar, B. and Chokkalingam, A. (1986) A note on supercritical carbon dioxide decaffeination of coffee. *J. Food*. *Sci*. *Technol*., 23, 326–328.

33. Coultate, T.P. (2009) Food: the chemistry of its components. The Royal Society of Chemistry, Cambridge, UK.

34. Woolfolk, C.A. (1975) 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.