Iranian Journal of Microbiology
Volume 6 Number 6 (December 2014) 428-436

ORIGINAL ARTICLE

Use of Taguchi methodology to enhance the yield of caffeine removal with growing cultures of *Pseudomonas pseudoalcaligenes*

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Received: March 2014, **Accepted:** July 2014

ABSTRACT

Background and Objectives: Microbial caffeine removal is a green solution for treatment of caffeinated products and agro-industrial effluents. We directed this investigation to optimizing a bio-decaffeination process with growing cultures of *Pseudomonas pseudoalcaligenes* through Taguchi methodology which is a structured statistical approach that can be lowered variations in a process through Design of Experiments (DOE).

Material and Methods: Five parameters, i.e. initial fructose, tryptone, Zn^{+2} ion and caffeine concentrations and also incubation time selected and an L16 orthogonal array was applied to design experiments with four 4-level factors and one 3-level factor ($4^4\times 1^3$). Data analysis was performed using the statistical analysis of variance (ANOVA) method. Furthermore, the optimal conditions were determined by combining the optimal levels of the significant factors and verified by a confirming experiment. Measurement of residual caffeine concentration in the reaction mixture was performed using high-performance liquid chromatography (HPLC).

Results: Use of Taguchi methodology for optimization of design parameters resulted in about 86.14% reduction of caffeine in 48 h incubation when 5g/l fructose, 3 mM Zn^{+2} ion and 4.5 g/l of caffeine are present in the designed media. Under the optimized conditions, the yield of degradation of caffeine (4.5 g/l) by the native strain of *Pseudomonas pseudoalcaligenes* TPS8 has been increased from 15.8% to 86.14% which is 5.4 fold higher than the normal yield.

Conclusion: According to the experimental results, Taguchi methodology provides a powerful methodology for identifying the favorable parameters on caffeine removal using strain TPS8 which suggests that the approach also has potential application with similar strains to improve the yield of caffeine removal from caffeine containing solutions.

Keywords: Caffeine removal, Process optimization, *Pseudomonas pseudoalcaligenase*, Taguchi methodology

INTRODUCTION

Caffeine (1, 3, 7-trimethyl xanthine) which its chemical formula is $C_8H_{10}N_4O_2$ belongs to a family of occurring methylxanthines. It is found in the leaves and fruits of thirteen plant families such as tea (*Camellia* species), coffee (*Coffea* species), cocoa (*Theobroma cacao*) and so on (1). Caffeine is the central nervous system (CNS) stimulant and its hyper-consumption can bring about insomnia (2). In humans, caffeine is a non-selective antagonist for adenosine receptors which acts as a phosphodiesterase inhibitor and exerts its stimulant effects by raising the cytosolic cAMP concentration (3). Several clinical studies suggest that caffeine has several effects on cardiac arrhythmias, cholesterol and blood pressure. On the other hand, there is a relevance between

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consumption of caffeinated beverages and risk of coronary heart disease (4). Moreover, coffee intake in men and women can cause raised calcium excretion in the urine (5). Detrimental effects of caffeine not limited to health precinct. It may also affect the germination of seed and fertility of agricultural land (6). Wastewaters resulting from the coffee industry might enter the adjacent ground and surface waters and make a big cause of pollution of rivers, lakes and environment (7).

Although the coffee pulp is rich in nutritional compounds such as proteins, carbohydrates and minerals and it has a potential to be applied for use in animal feed. However, the presence of several anti-nutritional compounds such as caffeine, polyphenols and tannins not allow the coffee pulp to be used as a domestic animal feed (8, 9).

According to the above mentioned topics, two perspectives can be described for removing caffeine from caffeinated foods and coffee processing industrial wastewater: (i) The presence of caffeine in human's diet can threats health due to its deleterious effects and (ii) the existence of caffeine in industrial effluents that associated with the coffee industry leads to environmental challenges. Over the past decades, several conventional methods such as solvent extraction method (10), Subcritical water extraction (11) and Supercritical carbon dioxide decaffeination (12) has been proposed to remove the caffeine from caffeine-containing solutions. But none of the techniques couldn't remove caffeine efficiently and problems such as high toxicity, lack of economic efficiency and non-specific removal of caffeine limit on use of the methods. Considering the inefficiency of traditional decaffeination methods, researcher are focusing on the applicability of microbial strains in the removal of caffeine. Different genera of filamentous fungi such as *Penicillium* (13), *Aspergillus* (14) and *Fusarium* (15) and yeasts belonging to the species *Saccharomyces cerevisiae* (16) and *Trichosporon asahii* (17) as well as several species of bacteria belonging to *Alcaligenes* spp. (18) and *Pseudomonas* spp. (19-21) has been reported to degrade caffeine in different conditions of media.

Over the past decades, statistical experimental methods have emerged as a robust tool in the industrial process improvement. Taguchi method is a structured approach that can be lowered variations in a process through Design of Experiments. The basic principle of the Taguchi study is to test the effects of many different parameters by varying them simultaneously rather than changing one factor at a time. The design allows fast and accurate estimation of the individual factors having main effects and select the best combination of the factors that will reach optimal conditions. More recently, Taguchi methodology as a powerful statistical approach has been applied to get the most parameters for improving of biotechnological processes including food-processing, microbial bio-transformation, microbial fermentation and wastewater treatment (22-25). As far as we know, no study has been reported on the application of Taguchi experimental design to optimize the caffeine removal of caffeine-containing media. The current study was conducted for optimizing a bio-decaffeination process with growing cultures of *Pseudomonas pseudoalcaligenes* through the Taguchi methodology.

MATERIALS AND METHODS

Microorganism and chemicals. The native strain TPS8 isolated from soil samples collected from tea cultivation fields in northern regions of Iran for its capability to use caffeine as the sole carbon and energy source (21). The strain was identified to the species level as *Pseudomonas pseudoalcaligenes* by using combining its morphological and biochemical characteristics with information derived from its 16S rRNA gene sequence and deposited in the NCBI database under GenBank accession number KF414528**.** *P. Pseudoalcaligenes* strain TPS8 were recovered from 15% glycerol stocks stored at −20° C before use. It was preserved in nutrient broth medium (0.3% beef extract, 0.5% peptone, 0.5% NaCl, pH 7) at 4º C. Caffeine (>99% purity) used for decaffeination experiments was purchased from Sigma Chemicals (St. Louis, Missouri, USA). Fructose and tryptone were prepared from Difco Company (Detroit, MI, USA). Zinc sulfate was purchased from Merck (E. Merck, Darmstadt, Germany). HPLC Grade acetonitrile and methanol were obtained from Merck, Germany. All other chemicals used were of analytical grade and commercially available.

Culture condition. A loop full from an overnight culture of *P. Pseudoalcaligeanse* TPS8 growing on nutrient agar plate containing 3g/l Beef Extract, 5 g/l Peptone and 15 g/l agar was used to inoculate 50 ml of a minimal M9 medium containing (g/l):

Tabel 1. Factors and their levels applied in the Taguchi experimental design for caffeine removal by *P. pseudoalcaligenes* TPS8.

Serial number	Factor	Level 1	Level 2	Level 3	Level 4
1	Fructose (g/l)	0	1	3	5
2	Tryptone (g/l)	0	1	3	5
3	Zn^{+2} ion (mM)	0	1	3	5
4	Caffeine (g/l)	0.5	2.5	4.5	6.5
5	Time incubation (h)	28	38	48	-

$MgSO_4.7H_2O$ 0.5, $CaCl_2$ 0.015 and NaCl 0.5 and aerobically incubated on a rotary shaker (150 rpm) at 28º C (26). The basal medium was buffered with 0.1 M potassium phosphate buffer (pH 7.2). The medium composition was changed in accordance with the taguchi experimental design. All experiments were carried out in triplicates.

Screening methodology. Single factor optimization was applied to screen design parameters that significantly influenced the caffeine removal by use of growing cultures of *P. pseudoalcaligenes* strain TPS8. For this purpose, various carbon sources including fructose, galactose, glucose, glycerol and sucrose at a concentration of 1 g/l, different nitrogen sources (ammonium chloride, casein, peptone, tryptone, yeast extract and urea at a concentration of 1 g/l) and several metal ions (Cu^{+2}, Co^{+2}, Fe^{+2}, Mn^{+2} and Zn^{+2} at a concentration of 1 mM) were investigated by changing one factor at a time while keeping the others constant. Different media components (carbon/nitrogen sources and metal ions) were added to the buffered M9 minimal media supplemented with 2.5 g/l of initial caffeine concentration. The culture media were incubated aerobically 48 h at 28º C with shaking speed 150 rpm. In each case, the caffeine removal yield was calculated.

Taguchi experimental design. Taguchi experimental design establishes systemic, simple and cost-effective methodology for the optimization of the significant parameters with a set of well-defined experiments (27). For the present study, an L16 orthogonal array was used to design experiments with four 4-level factors and one 3-level factor ($4^4\times 1^3$). Factors are designated in the column in random order. The results of the evaluations were analyzed singly. Data analysis was performed by use of the statistical analysis of variance (ANOVA) method. Furthermore, the optimal conditions were determined by combining the optimal levels of the significant factors and verified using a confirmation test. All calculations were analyzed using Qualitek-4 software (W32b, Nutek, Inc., Michigan, USA).

Assay of caffeine degradation. Measurement of residual caffeine concentration in the reaction mixture was performed in a high-performance liquid chromatography system with a C18 column (5 µm, 250×46 mm) and equipped with a ultraviolet detection at 278 nm. A mixture of water-acetonitrile in a ratio of 75:15 (v/v), as the mobile phase, at a flow rate of 1 ml/min and with an injection volume of 20 µl isocratically was run. Under these conditions, the retention time of caffeine was 7.4 min. The percentage of caffeine removal was estimated by the formula: % caffeine removal= initial caffeine concentration (g/l) - residual caffeine concentration (g/l)/ initial caffeine concentration (g/l)× 100 (20).

RESULTS

Analysis of experiments and results. To screen the major components affecting microbial caffeine degradation, in preliminary experiments, various carbon and nitrogen sources, and several metal ions were tested for their suitability to bio-decaffeination experiments and selected through the one-factor-at-a-time method (OFAT). The results revealed that the best combination factors for caffeine degradation was fructose, tryptone and Zn^{+2} ion (data not shown). These factors were chosen for the Taguchi optimization process.

After selecting the best combination of parameters for caffeine removal using growing cultures of *P. pseudoalcaligenes* TPS8, process optimization using the Taguchi methodology was carried out. The Taguchi methodology is to evaluate the main effect of individual design factors on caffeine degradation, management of interactions between control factors, determining the optimum process conditions and final measurement of caffeine removal rate under the

Tabel 2. L16 orthogonal array of Taguchi experimental design for optimizing the removal of caffeine using *P. pseudoalcaligenes* TPS8.

Serial no.	1 (Fructose)	2 (Tryptone)	3 (Zn^{+2} ion)	4 (Caffeine)	5 (Time incubation)	Average Caffeine degradation (%)
1	1	1	1	1	1	38.6
2	1	2	2	2	2	44.4
3	1	3	3	3	3	66.3
4	1	4	4	4	1	47.4
5	2	1	2	3	1	59.5
6	2	2	1	4	3	62.3
7	2	3	4	1	2	50.1
8	2	4	3	2	1	53.9
9	3	1	3	4	2	79.3
10	3	2	4	3	1	71.8
11	3	3	1	2	1	59.5
12	3	4	2	1	3	54.0
13	4	1	4	2	3	78.3
14	4	2	3	1	1	60.9
15	4	3	2	4	1	55.4
16	4	4	1	3	2	82.7

optimal experimental conditions.

In this regard, five factors viz., initial fructose, tryptone, Zn^{+2} ion and caffeine concentrations and also incubation time selected (Tabel 1) and a standard orthogonal array L16 (Tabel 2) with 15 degrees of freedom was employed to study four factors in 4-level designs and one factor in 3-level designs. The L and the subscript (16) equal the Latin square and the number of experimental runs, respectively. The levels of the factors examined and the layout of the L16 Taguchi orthogonal array is presented in Table 1 and 2. As seen in Table 2, according to the combinations of the selected factors, the efficiency of caffeine removal yield ranged from 38.6% to 82.7%. The influence of selected level factors on caffeine degradation rate is shown in Fig. 1. Higher rate of caffeine degradation was obtained with level three of caffeine, Zn+2 and time of incubation and at level four for fructose and also with level one of tryptone (Fig. 1). Therefore, by looking at the effects of each individual factor separately (their main effects), the overall effect of the factors on biological removal of caffeine using *P. pseudoalcaligenes* TPS8 can be distinguished. The calculated interactions between process parameters affecting caffeine removal are shown in Tabel 3. The estimated interaction serverity index (SI) of the factors under investigation helps us to study the impact of two individual factors at different levels of the interactions (28).

From Table 3, it is clear that the highest interaction (SI=68.14%) was found between tryptone and incubation time and the lowest interaction (SI=2.62%) was observed between fructose and caffeine. It is interesting to note that tryptone and time of incubation individually have a relatively small main effects while in combination with other factors, they showed the highest severity index. The results suggest that the effect of one factor to get the most removal of caffeine depends the levels of the other factors. Taguchi method has a statistical tool including analysis of variance (ANOVA) which can be applied to validity of the experimental results and to determine the contribution of each individual factor to total experimental response. Analysis of data using the ANOVA method for determining of significant factors on caffeine removal was carried out and the results are illustrated in an ANOVA Table after pooling-up technique (Tabel 4).

The pooling-up approach was proposed by Taguchi for calculating the error variance and to determine significance of the process parameters. On the basis of the pooling results, initial fructose concentration, caffeine concentration, time of incubation and Zn^{+2} ion concentration were the most significant factors

Tabel 3. Estimation of interactions for different factors. Ten interactions between two factors have estimated by Qualitek-4 (W32b) software.

Serial number	Factors	Columns[a]	SI (%)[b]	col[c]	Opt[d]
1	Tryptone × Time	2× 5	68.14	7	(4, 2)
2	Tryptone × Caffeine	2 × 4	63.71	6	(4, 3)
3	Zn^{+2}× Time	3 × 5	60.96	6	(1, 2)
4	Tryptone × Zn^{+2}	2 × 3	43.99	1	(4, 1)
5	Zn^{+2}× Caffeine	3 × 4	34.58	7	(1, 3)
6	Caffeine × Time	4 × 5	16.51	1	(3, 2)
7	Fructose × Time	1 × 5	10.07	4	(4, 2)
8	Fructose × Zn^{+2}	1 × 3	9.75	2	(4, 1)
9	Fructose × Tryptone	1 × 2	3.40	3	(4, 4)
10	Fructose × Caffeine	1 × 4	2.62	5	(4, 3)

[a] Columns represent the column locations to which the interacting factors are assigned. [b]SI-interaction severity index (100% for 90º angle between the lines, 0% for parallel lines. [c]Col-shows column that should be reserved if this interaction effect was to be studied (2-evel factors only). [d]Opt-indicates the factor levels desirable for the optimum conditions.

for decaffeination experiments, respectively. The confidence levels for fructose, caffeine, incubation time and Zn^{+2} ion , 99.84%, 99.72%, 99.27% and 98.55%, respectively. The remaining factor namely tryptone had the least significant and was pooled for this aim. Under these conditions, the yield of caffeine removal was estimated with only the more significant of the selected factors. ″Bigger to Better″ analysis was performed to find the optimum conditions for various factors and their performance to enhance caffeine removal effeiency (Tabel 5). Based on the obtained results, as shown in Tabel 5, factors such as initial caffeine concentration and fructose concentration play a more significant role in caffeine removal experiments than the other selected factors. The results indicate that the yield of expected caffeine removal under the optimum conditions was calculated as 88. 897%. With these selected level factors, the total contribution from all factors and the current grand average performance were 28.621% and 60.275%, respectively.

Confirming experiment. To confirm the predicted optimum conditions, bio-decaffeination experiments using growing cultures of *P. Pseudoalcaligense* TPS8 were performed with the optimal levels of each individual factor in the following medium: fructose 5g/l, Zn^{+2} ion 3 mM, caffeine 4.5 g/l and time of incubation 48 h. According to the experimental results, the observed yield of caffeine removal under these optimal conditions was 86.14%. This indicated there was a good agreement between predicted and

Tabel 4. Analysis of Variance (ANOVA) after Pooling-up technique.

Serial no.	Factors	DOF (f)	Sum of Sqrs. (S)	Variance (V)	F-Ratio (F)	Pure Sum (S)	Percent (P %)
1	Fructose	3	1017.032	339.01	46.876	995.336	40.469
2	Tryptone	(3)	(80.42)		POOLED	(CL=67.25%)	0.000
3	Zn+2 ion	3	297.89	99.296	13.73	276.195	11.229
4	Caffeine	3	744.692	248.23	34.324	722.996	29.396
5	Time incubation	2	312.181	156.09	21.583	297.717	12.105
Other/Error		4	87.651	21.913			6.801
Total		15	2459.45				100.000%

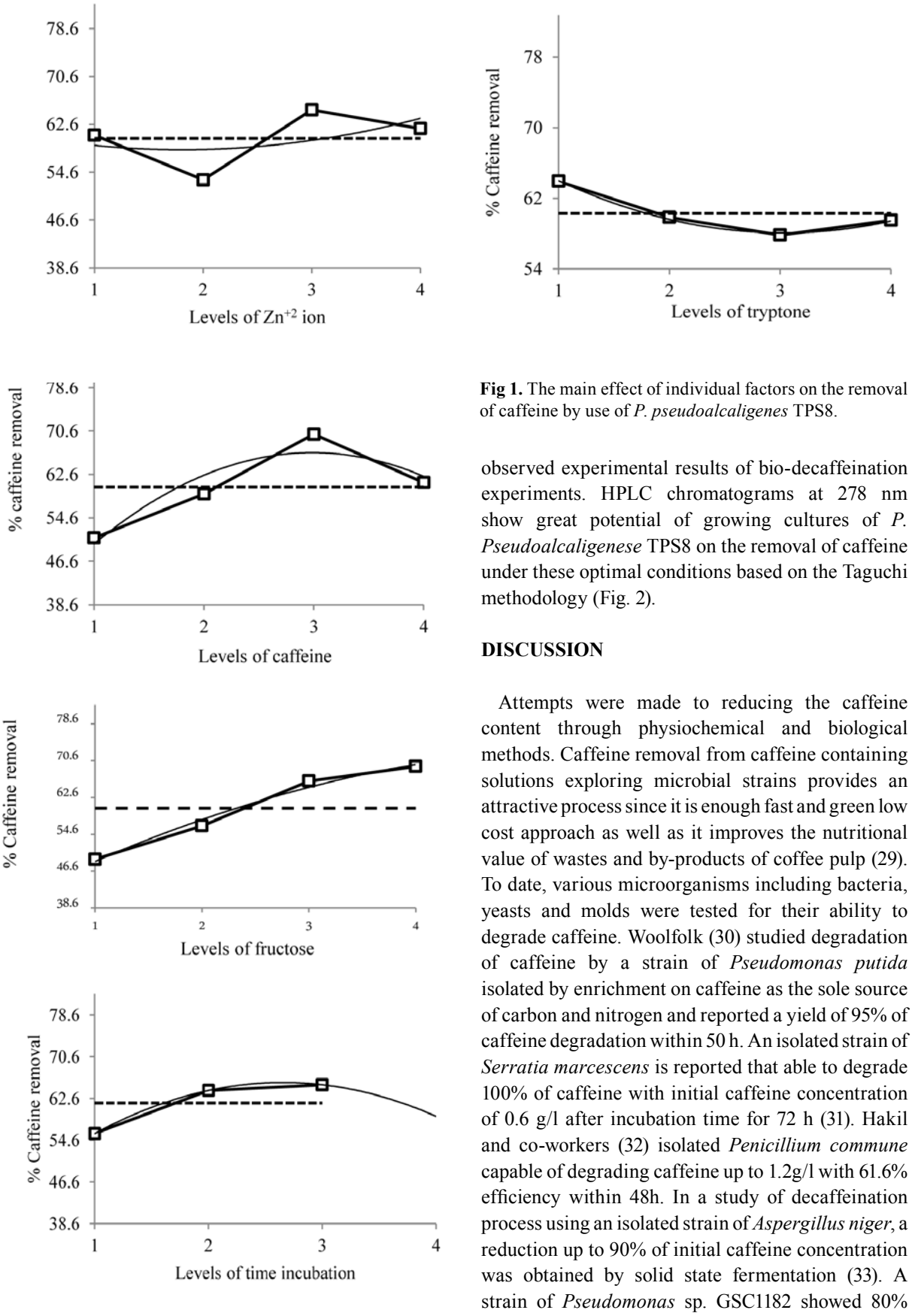

Fig 1. The main effect of individual factors on the removal of caffeine by use of *P. pseudoalcaligenes* TPS8.

observed experimental results of bio-decaffeination experiments. HPLC chromatograms at 278 nm show great potential of growing cultures of *P. Pseudoalcaligenese* TPS8 on the removal of caffeine under these optimal conditions based on the Taguchi methodology (Fig. 2).

DISCUSSION

Attempts were made to reducing the caffeine content through physiochemical and biological methods. Caffeine removal from caffeine containing solutions exploring microbial strains provides an attractive process since it is enough fast and green low cost approach as well as it improves the nutritional value of wastes and by-products of coffee pulp (29). To date, various microorganisms including bacteria, yeasts and molds were tested for their ability to degrade caffeine. Woolfolk (30) studied degradation of caffeine by a strain of *Pseudomonas putida* isolated by enrichment on caffeine as the sole source of carbon and nitrogen and reported a yield of 95% of caffeine degradation within 50 h. An isolated strain of *Serratia marcescens* is reported that able to degrade 100% of caffeine with initial caffeine concentration of 0.6 g/l after incubation time for 72 h (31). Hakil and co-workers (32) isolated *Penicillium commune* capable of degrading caffeine up to 1.2g/l with 61.6% efficiency within 48h. In a study of decaffeination process using an isolated strain of *Aspergillus niger*, a reduction up to 90% of initial caffeine concentration was obtained by solid state fermentation (33). A strain of *Pseudomonas* sp. GSC1182 showed 80% degradation of caffeine in 48h when caffeine was

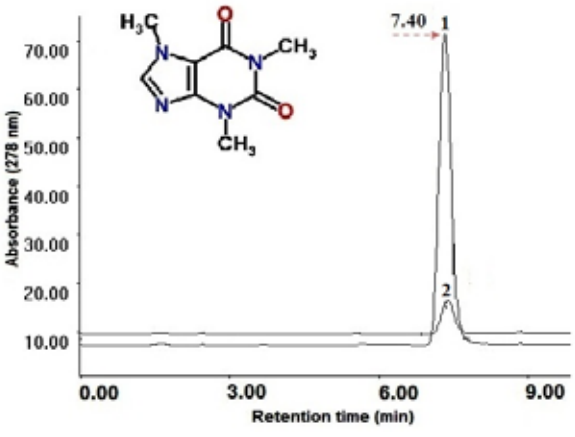

Fig 2. Overlaid HPLC chromatograms at 278 nm showing caffeine removal effiency by *P. pseudoalcaligenes* TPS8 under optimal conditions after 0 (chromatogram 1) and 48 (chromatogram 2) hour incubation time.

used as the sole carbon and nitrogen source (34). Much investigation was performed on optimization of environmental and physiological parameters for efficient biodegaradation of caffeine. Dash and Gummadi (35) developed a bio-decaffeination process optimization for biodgradation of caffeine by *Pseudomonas* sp. NCIM5235 by means of full factorial central composite design. Under the optimum process conditions, the rate of degradation of caffeine has been increased from 0.18 to 0.29 g/l which is 1.6 fold higher than the normal rate. Response surface methodology was employed to optimize the removal efficiency of caffeine using immobilized *Pseudomonas* sp. cells (36).

The initial rate of degradation of caffeine with this immobilized cell was 0.08g/l/h and after optimization increased to 0.15g/l/h. Using *Trichosporn asahii*, a yeast species from caffeine contaminat soil, 100% of degradation of caffeine (2g/l) was achieved within 96 hours in the presence of 5 g/l of sucrose under process optimization by one factor at the time approach (17). Growing cultures of *Pseudomonas stutzeri* Gr21ZF showed 59% of 1.2 g/l caffeine degradation in 24h without further optimization process whereas Optimizing of the process parameters using Plackett-Burman design methodology increased the caffeine degradation up to 86% (37).

In the current investigation, a two step optimization procedure was applied for improving degradation of caffeine by the native isolated *P. pseudoalcaligenes* strain TPS8 under growing cultures. Firstly, the one-factor at the time methodology was used to screen the significant factors. The methodology of Taguchi design was employed in the second step to determine the optimum levels of the selected factors to maximize caffeine degradation efficiency. In the preliminary optimization, the effect of different carbon and nitrogen sources and metal ions affecting degradation of caffeine were studied by one-factor-at-a-time (OFAT) experiment. The addition of external carbon and nitrogen sources as cosubstrates are essential for the growth of *P. psedoalcaligenes* TPS8 and also for improvement degradation of caffeine by the bacterial strain. Metal ions influence the activity of enzymes involved in the removal of caffeine by either increasing or inhibiting their activity.

After initial optimization studies, fructose, tryptone and Zn^{+2} ion found to the best combination of variables for bio-decaffeination experiments and selected for further optimization through Taguchi approach. Concentrations of the three media ingredients (fructose, tryptone and Zn^{+2} ion) as well as initial caffeine concentration and time of incubation were optimized using Taguchi methodology to predict the maximal removal of caffeine under growing cultures of *P. psedoalcaligenes* strain TPS8.

Tabel 5. Optimum conditions and their performance in bio-decaffeination experiments for maximum caffeine removal using *P. pseudoalcaligenes* TPS8 after pooling.

Serial number	Factors	Level description	Level	contribution
1	Fructose	5 g/l	4	9.049
3	Zn^{+2} ion	3 mM	3	4.824
4	Caffeine	4.5 g/l	3	9.799
5	Time incubation	48 h	3	4.950

Total contribution from all factors: 28.621 (% caffeine removal)
Current grand average of performance: 60.275 (% caffeine removal)
Expected result at optimum condition: 88. 897 (% caffeine removal)

An L16 orthogonal array has been employed to accommodate the experiments. The results indicated the initial concentrations of caffeine, fructose, Zn^{+2} ion and incubation time could significantly affect the bio-decaffeination experiments. The optimal combination of the significant factors also validate by performing the confirmation experiments. According to the obtained results, the yield of caffeine removal under the optimal conditions was 86.14%.

Our previous study (21) showed that growing cultures of *P. pseudoalcaligenes* TPS8 reduced caffeine in 80.2% yield with incubation for 72h in minimal salt medium with 2.5 g/l caffeine as the sole carbon and nitrogen source. However, with the increase of caffeine concentration up to 4.5 g/l, the yield of caffeine removal decreased significantly and a maximum removal of caffeine of 15.8% was obtained after 48 h incubation without further optimization. Application of Taguchi methodology for optimization of design parameters resulted in about 86.14% reduction of caffeine in 48 h incubation when 5g/l fructose, 3 mM Zn^{+2} ion and 4.5 g/l of caffeine are present in the same minimal media. Under the optimized conditions, the yield of degradation of caffeine (4.5 g/l) by the native strain of *P. pseudoalcaligenes* TPS8 increased from 15.8% to 86.14% which is 5.4 fold higher than the normal yield. These results demonstrated that growing cells of *P. pseudoalcaligenes* TPS8 have greater capacity to tolerate and degrade caffeine under these optimal conditions as compared to non-optimized conditions as described in our previous study (21).

Based on the experimental results of this investigation, Taguchi orthogonal design approach provides a simple, systemic and powerful methodology for identifying the favorable parameters on caffeine removal using growing cells of *P. pseudoalcaligenes* strain TPS8 which proposes that the methodology also has potential application with similar strains to improve the yield of caffeine removal from caffeine containing solutions. This is the first investigation in which Taguchi experimental design was employed successfully to bio-decaffeination experiments.

ACKNOWLEDGMENT

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

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