



Removal of caffeine from industrial wastewater using *Trichosporon asahii*

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Abstract

Caffeine (1,3,7-trimethylxanthine), a natural alkaloid present mainly in tea and coffee products has been suggested as an environmental pollutant. Decaffeination is an important process for the removal of caffeine from coffee industrial wastes. In the present study, caffeine removal (through degradation) by yeast isolate, *Trichosporon asahii* immobilized on various conventional matrices (sodium alginate, carboxymethyl cellulose, chitosan, agar and agarose) was investigated using the method of entrapment. The biofilm forming ability of *T. asahii* was monitored by atomic force microscopy and scanning electron microscopy. Exopolysaccharide produced by *T. asahii* biofilm was characterized by FT-IR spectroscopy and HPLC analysis. Caffeine removal from coffee processing industrial effluent was found to be 75 and 80 % by alginate immobilized yeast and yeast biofilm formed on gravels over a period of 48 hr in batch mode. Effectiveness of the process was also tested involving the continuous - flow column studies.

Key words

Biofilm, Caffeine removal, *Trichosporon asahii*, Immobilization, Entrapment

Introduction

Caffeine is a major pharmacologically active ingredient naturally occurring in coffee, cocoa beans, tea leaves, cola nuts etc. Caffeine is released in the surrounding water bodies and has been detected in ground water, surface water, and also in wastewater effluents having high concentration (~10g l⁻¹) (Buerge *et al.*, 2003; Weigel *et al.*, 2004; Glassmeyer *et al.*, 2005).

Caffeine has been suggested as a chemical indicator of environmental pollution 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 (Chen *et al.*, 2008). Reports also showed that caffeine is toxic to most of the aquatic organisms such as *Ceriodaphnia dubia* (LC₅₀ = 60 mg l⁻¹), *Pimephales promelas* (LC₅₀ = 100 mg l⁻¹), *Chironomus dilutus* (midge) (LC₅₀ = 1.230 g l⁻¹), *Xenopus laevis* (LC₅₀ = 0.35 mg ml⁻¹) (Moore *et al.*, 2008) and coral algae (Pollack *et al.*, 2009). Amphibians may also be affected by exposure to caffeine

that appears to be widespread in aquatic ecosystems (Fraker and Smith, 2004). To make the various water sources free from this xenobiotic, removal of caffeine becomes a necessary step in treatment of caffeine containing wastewaters (Dash and Gummadi, 2007). Soil fertility is affected in the presence of caffeine and it also inhibits seed germination and growth of seedlings (Batish *et al.*, 2008). The presence of caffeine in coffee pulp and husk restricts its use as animal feed (Mazzafera *et al.*, 1994).

Conventional methods *viz.* solvent, water (Senol and Aydin, 2006) and supercritical carbon dioxide extraction (Kim *et al.*, 2008) used for caffeine removal are either costly or it involves the usage of toxic organic chemicals. Caffeine is extracted with solvents such as trichloroethylene, methylene chloride or some other similar chlorinated compounds. This method has to meet stringent environmental restriction while discarding the solvent (Gokulakrishnan *et al.*, 2005). The disadvantage of supercritical carbon dioxide extraction is the high cost equipment due to the usage of high pressure and also it needs large quantity of CO₂ which is very costly. Moreover the use of membrane or carbon filters in caffeine removal will be very expensive and the commercialization of

the process becomes less viable (Gokulakrishnan *et al.*, 2005). Biological method involving microbes for caffeine removal has been considered more suitable than currently used chemical methods. There are reports on bacteria and fungi, viz. *Pseudomonas* sp. (Gokulakrishnan *et al.*, 2007), *Serratia* sp. (Mazzafera *et al.*, 1994), *Klebsiella* and *Rhodococcus* sp. (Madyastha and Sridhar, 1998), *Aspergillus* and *Penicillium* sp. (Hakil *et al.*, 1998) which have shown their potentiality for degrading caffeine. But reports are scanty regarding the potentiality of yeast as caffeine degrader.

The use of microorganisms such as bacteria, yeast, fungi and algae in freely suspended state is limited for degradation of toxic compounds owing to some disadvantages including small particle size, possible clogging and low mechanical strength of the biomass (Godjevargova *et al.*, 2004). The efficacy of biodegradation is often improved when immobilized microbial cells are used. Immobilized microbial cells have several potential advantages over free cells for the bioremediation of anthropogenic wastes (Wang *et al.*, 1997; Baskaran and Nemat, 2006). Moreover, the immobilized cells are less likely to be adversely affected by predators, toxins or parasites compared to free cells (Prabua and Thatheys, 2007).

Microorganisms often switch from a free-living life style to a surface adapted, multicellular organization known as biofilm. It is a kind of immobilization of microorganisms in a solid matrix and can be applied for bioremediation of wastewaters (Costerton *et al.*, 1995). For bioremediation of xenobiotic compounds, biofilm reactors became a focus of interest for researchers during the last few decades (Singh *et al.*, 2006). So far, there is no report regarding the application of immobilized yeast and yeast biofilm for the removal of caffeine from industrial wastewater through degradation.

In our previous study, we have reported the caffeine degrading potential of free cells of yeast species, *Trichosporon asahii* isolated from caffeine contaminated soil (Lakshmi and Das, 2010; 2011). The objectives of the present study are (i) to investigate the caffeine degrading efficiency of *T. asahii* immobilized in various conventional matrices (ii) to study the biofilm forming ability of *T. asahii* on PVC strips through microscopic analysis (iii) to quantify and characterize the exopolysaccharides (EPS) produced by *T. asahii* biofilm and (iv) to investigate caffeine removal from industrial wastewater using alginate immobilized *T. asahii* and *T. asahii* biofilm formed on gravels.

Materials and Methods

Yeast and growth conditions: The yeast species, *Trichosporon asahii* was isolated from caffeine contaminated soil under coffee cultivation area, collected

from Coffee Board, Yercaud, India. The yeast was identified to the species level using VITEK 2 compact yeast card reader with the software version: 03.01 from Council for Food Research and Development (CFRD), Kerala, India. Growth media viz. (i) Caffeine liquid medium (CLM) containing (g l⁻¹): 0.8 K₂HPO₄; 0.2 KH₂PO₄; 0.2 MgSO₄.7H₂O; 0.1 CaCl₂.2H₂O; 0.005 FeSO₄.7H₂O; 0.2 yeast extract; 2.0 sucrose and 2.0 caffeine and (ii) Yeast Extract Peptone Dextrose (YEPD) medium containing (g l⁻¹): 10 yeast extract; 20 peptone and 20 dextrose, were used. For solid medium 20 g l⁻¹ agar was added to CLM. The pH and temperature was maintained at 6.5 and 28 °C.

Industrial wastewater: Coffee processing industrial wastewater was collected from Coffee Board, Yercaud, India. The physico-chemical parameters such as pH, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), Total Dissolved Solids (TDS) and Total Suspended Solids (TSS) were estimated following the standard methodologies of APHA (2005). The initial caffeine concentration in the effluent was measured using UV-visible spectrophotometer.

Methods for immobilization of yeasts : The concentration of immobilization matrices used was 3 % and the inoculum size was 4 g w. wt. l⁻¹. The preparation of immobilized beads was carried out under sterilized condition. The mixture of sodium alginate and yeast suspension was gently dropped into CaCl₂ solution (0.2 M) to form alginate beads. To enhance the mechanical stabilities, immobilized alginate beads were cured in 0.2 M CaCl₂ solution for 2 hr. The mixture of CMC solution and yeast suspension was gently dropped into FeCl₃ solution (0.05 M) to form CMC beads. To enhance the mechanical stabilities, immobilised beads were cured in the FeCl₃ solutions for 1 hr. Chitosan solution was prepared in 100 ml of 1 % acetic acid and thoroughly mixed with yeast suspension. The chitosan-yeast mixture was gently dropped to an NaOH solution (8%) for coagulation and the chitosan beads were formed. The immobilized beads were separated from the solution after 30 min and washed twice with 200 ml of sterile distilled water for 15 min.

Agar solution was melted by heating. Yeasts suspension was mixed with 100 ml of the agar solution (cooled to temperature ≤ 40 °C). Mixture of agar and yeast suspension was gently dropped into sterile petri plates coated thin layer of refined sunflower oil and allowed to solidify for 30 min at 4 °C. The solidified immobilized agar block was cut into equal size cubes (~2 mm). The immobilized agarose blocks were prepared following the method as described for agar.

Screening of different matrices for caffeine degradation:

To screen the best matrix for immobilization and caffeine degradation, yeast cells immobilized on various matrices were transferred into 50 ml of CLM containing 2 g l⁻¹ caffeine. The pH of the medium was adjusted to 6.5 and temperature

maintained at 28 °C on a rotary shaker at 120 rpm. At regular time intervals, samples were collected and degradation was monitored. The above procedure was also followed for studying the biodegradation of caffeine by free cells by inoculating free cells of *T. asahii* (4 g w.wt. l⁻¹) to the caffeine liquid medium containing 2 g l⁻¹ caffeine and incubated under same conditions stated above.

Analytical method for caffeine degradation: Caffeine from culture broth was extracted using chloroform as the solvent. A control system was incubated with substrates without immobilization of yeast to monitor abiotic losses of the caffeine substrate. The degradation of caffeine was expressed as the percentage of caffeine degraded in relation to the amount of the remaining fractions in the appropriate abiotic control samples. Caffeine concentration was estimated by UV-visible spectrophotometer (Hitachi U-2800). Absorbance was measured at 253 nm. Percentage of caffeine degradation was calculated by dividing the subtracted value from initial and residual caffeine concentration with initial caffeine concentration and multiplying with 100.

Growth study of free and immobilized yeast cells: Growth of free and immobilized yeast cell in different matrices, were monitored by total plate count (TPC) method. Immobilized beads were homogenized with phosphate-buffer (pH 7.0) and 0.1 ml of aliquots were plated on YEPD agar plates. The viability of free yeast cells were estimated by plating aliquots from CLM broth culture onto YEPD agar plates and incubated for 72 hr.

Biofilm formation and quantification: Biofilm formation assay by crystal violet (CV) staining in 96-well microtiter plate was chosen to assess cell attachment and formation of biofilms using a modified protocol from O'Toole and Kolter (1998). Microtiter plates were inoculated by adding 1.5 µl of the inoculum at different time intervals. Plates were incubated at 28 °C and 150 rpm. After incubation, liquid supernatants were carefully removed using a pipette, and wells were rinsed with 180 µl of phosphate-buffered saline (PBS). The attached biomass was stained with 180 µl of a filtered CV solution. The wells were then rinsed three times each with 200 µl PBS and were airdried. The remaining CV stain was solubilized in 180 µl of absolute ethanol, and the light absorbance was determined at 490 nm with a Model 680 Microplate Reader (Bio-Rad Laboratories Ltd, UK). Biofilm formation was scored based on an inoculum-free medium control, and the ratios of $A_{\text{biofilm}}/A_{\text{control}}$ are presented.

The sequence of biofilm formation was monitored using atomic force microscopy (AFM) and scanning electron microscopy (SEM). Images of the biofilms formed by yeast species on polyvinyl chloride (PVC) strips (1 cm²), were obtained using AFM following the procedure of Lal *et al.* (2010). The sample for SEM was prepared following the

method of Hawser and Douglas (1994) with minor modification. Briefly, yeast biofilms formed on PVC pieces (1 cm²) were fixed with 2.5 % glutaraldehyde in phosphate buffer for 2 hr at room temperature. They were then dehydrated in ethanol series (50, 80, 90 and 100 %). All samples were dried to critical point by polaron critical point drier, gold coated and viewed under SEM.

Quantification and characterization of exopolysaccharides (EPS): Exopolysaccharides were quantified following the method of Wehland and Bernhard (2000). Yeast cells grown on polyvinyl chloride (PVC) pieces (1 cm²) for 48 hr at 28 °C were harvested by scraping and resuspended in 5 ml of phosphate-buffered saline. EPS was separated from the yeast cells by vortexing the sample for 3 min, followed by ultracentrifugation of the yeast suspension at 160,000 g for 45 min at 10 °C. The supernatant was removed and dialyzed in dd H₂O for 3 hr. Polysaccharides were quantitated by addition of 60 µl of an 80 % phenol solution to 2 ml of the dialyzed EPS. Then, 5 ml of concentrated sulfuric acid was added. The tubes were incubated at room temperature for 10 min and then shaken and incubated at 30 °C for 20 min. EPS content was estimated by measuring the absorbance at 490 nm and plotting on a standard curve of glucose.

Purified exopolysaccharides were characterized using FT-IR spectroscopy and HPLC analysis. The infrared analysis of the dialysed EPS was carried out by KBr pellet technique using FT-IR spectrophotometer (Perkin Elmer Spectrum 1). Purified dextran was used as a standard, and the spectrum was measured in the frequency range of 450-4000 cm⁻¹.

The carbohydrate composition in the polysaccharides was determined after hydrolysis with 2N H₂SO₄ for 8 hr at 105 °C. Determination of neutral sugars and glucuronic acid in the hydrolysate was done by using HPLC (Waters, Milford, MA, USA) with a symmetry C 18 column (4.6×150 mm) and detection with λ Absorbance Detector (245 nm). The mobile phase was a solvent consisting of acetonitrile and water in a ratio of 60: 40. The flow rate was 1 ml min⁻¹.

Formation of biofilm on gravels: The gravels were immersed in CLM medium for a period of two weeks to form yeast biofilm artificially. The biofilm formed on gravels were washed with phosphate buffer (pH 7.0) after 2 weeks of incubation to remove the loosely attached yeast cells. The washed gravels were then used for caffeine degradation experiments.

Degradation efficiency at different initial concentration of caffeine: The effect of initial caffeine concentration on degradation by *T. asahii* cells immobilized in sodium alginate and biofilm formed on gravels by *T. asahii* cells was studied over the initial concentration ranging from 2 to 12 g l⁻¹. The percentage of caffeine degraded was measured at different time intervals.

Degradation of caffeine present in coffee processing industrial effluent: Coffee processing industrial effluent was collected from Coffee Board, Yercaud, Tamil Nadu, India. The concentration of caffeine was determined by extraction with equal volume of chloroform and UV-Visible spectrophotometric analysis. Experiments on degradation of caffeine in the effluent were performed in batch mode using alginate immobilized yeast and yeast biofilm formed on gravel. The degradation of caffeine was monitored at regular time intervals using UV-Visible Spectrophotometer. All experiments were performed in triplicates under identical conditions of pH 6.5 and temperature maintained at 28°C and the data presented are mean of triplicates.

In order to demonstrate the practical application of operating conditions during the degradation process in the present study, column experiments were also conducted using caffeine containing industrial effluent and the efficiency of the process was checked. The effluent was fed through a glass column (3.0 cm i.d. and 15 cm long) packed with yeast biofilm formed on gravels at a bed height of 12 cm and flow rate of 1.0 ml min⁻¹ controlled by a peristaltic pump. The samples collected from the exit were analysed at regular intervals for caffeine degradation using UV-Visible Spectrophotometer.

Results and Discussion

The yeast species *T. asahii* was immobilized in various matrices viz. sodium alginate, CMC, chitosan, agar and agarose and the viability was checked over a period of 10 weeks. The yeast cells immobilized in sodium alginate was found to be more stable and retained maximum viability compared to other matrices (Fig. 1). Stability of the beads is important to maintain high conversion of substrate to product (Idris and Suzana, 2006). Use of alginate has many advantages including: non-toxic and non-irritant, ease of use and mix, cheap and good shelf life, good surface detail and setting time can be controlled with temperature of water used (Leo *et al.*, 1990). Sarma and Pakshirajan (2011) reported that sodium alginate bead showed high mechanical stability and cell viability of immobilized *Mycobacterium frederiks bergense* compared to chitosan and PVA immobilized beads. Similarly, in case of immobilized cells of *Bacillus subtilis* PE-11, Adinarayana *et al.* (2005) reported that sodium alginate was an effective and suitable matrix for higher alkaline protease productivity compared to other matrices viz., k-Carrageenan, agar-agar, polyacrylamide and gelatin.

Yeast cells immobilized in alginate showed more growth than those immobilized in other substrates (Table 1). This proved that alginate provided suitable support for the growth of yeast species in caffeine containing media. Table 1 showed that alginate immobilized yeast *T. asahii* showed higher percentage of caffeine degradation (75 %), compared to the free cells (70 %). Therefore, in this study

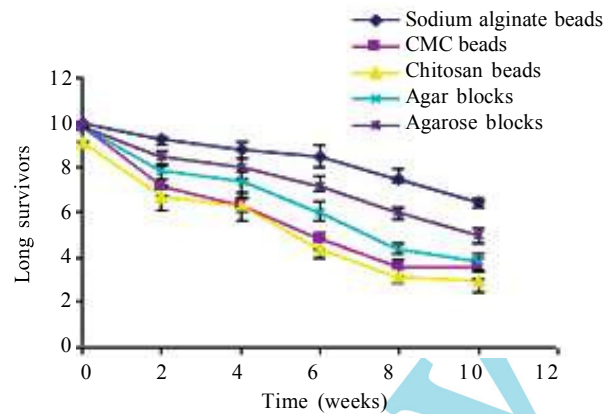


Fig. 1 : Time-course of changes in the viability of *T. asahii* immobilized in various matrices (T=28°C, pH=6.5)

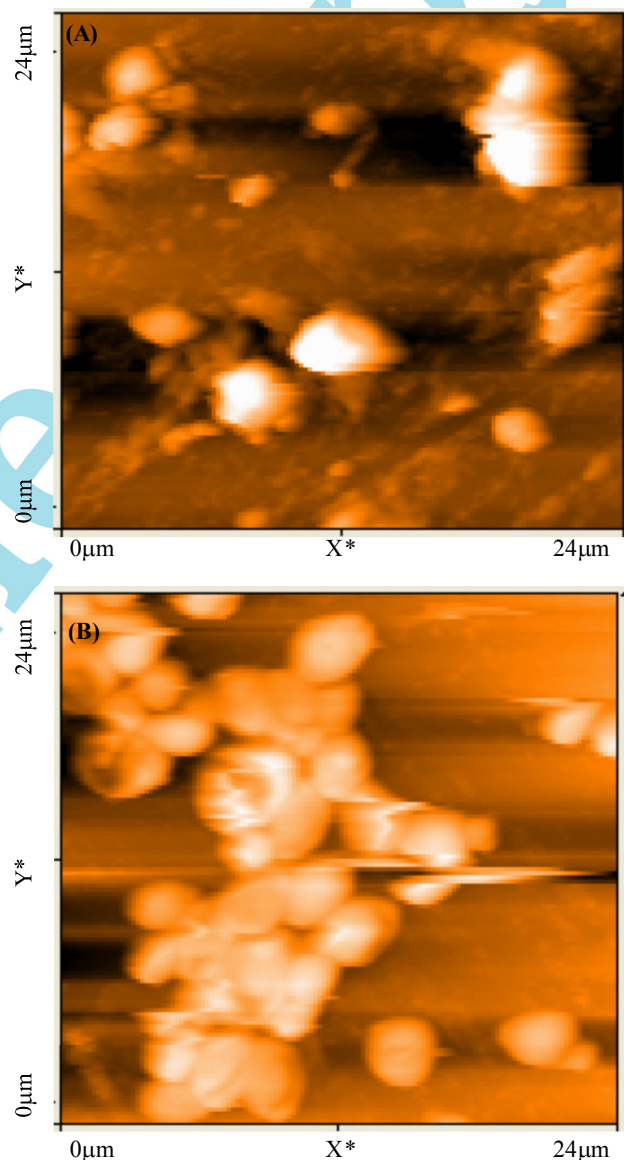


Fig. 2 : AFM images showing sequential biofilm formation by *T. asahii* on PVC strips during (a) 6 hr and (b) 12 hr

Table 1 : Biomass production and caffeine degradation potential of free and immobilized yeast cells grown in CLM (Initial caffeine concentration - 2 g l⁻¹; pH - 6.5; Temperature - 28 °C; Shaking speed – 120 rpm)

Inoculum	TPC (cfu ml ⁻¹)			Caffeine degradation (%)		
	24 hr	48 hr	54 hr	24 hr	48 hr	54 hr
Free cells	4.4 x 10 ⁸	6.5 x 10 ⁸	6.1 x 10 ⁸	64±3	70±1	70±2
Cells immobilized in						
Sodium alginate	5.0 x 10 ⁸	7.1 x 10 ⁸	6.8 x 10 ⁸	69±1	75±1	75±1
CMC	2.8 x 10 ⁸	4.4 x 10 ⁸	4.1 x 10 ⁸	58±2	61±1	61±2
Chitosan	1.9 x 10 ⁸	2.8 x 10 ⁸	2.5 x 10 ⁸	47±2	56±2	56±1
Agar	2.2 x 10 ⁸	4.0 x 10 ⁸	3.8 x 10 ⁸	54±3	60±3	60±1
Agarose	2.0 x 10 ⁸	3.0 x 10 ⁸	2.7 x 10 ⁸	49±1	58±3	58±2

Values are mean of replicate ± SD

we reported for the first time that it has been possible to develop immobilized yeast species *T. asahii* showing more caffeine degradation capacity compared to free cells.

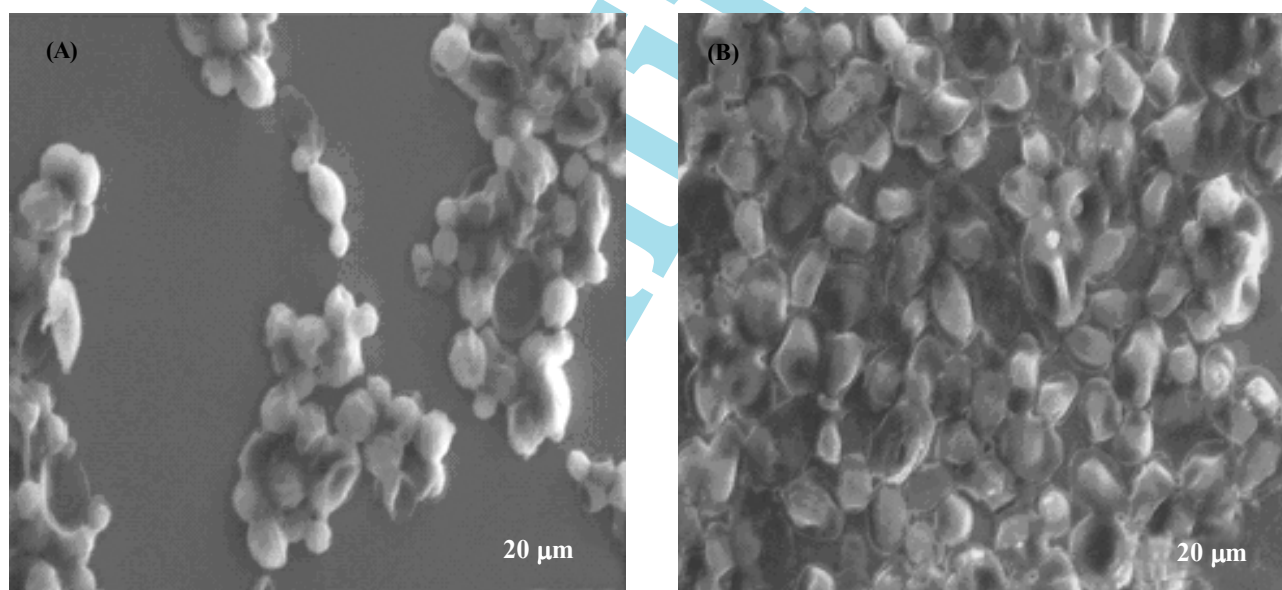
The adherence and subsequent biofilm formation by yeast cells were studied using crystal violet staining assay. The biofilm score of *T. asahii* was 8.2 (Average of $A_{\text{biofilm}}/A_{\text{control}}$ at 48 hr). It is evident that *T. asahii* was capable of single-species biofilm formation. The sequence of biofilm formation on PVC strips could be clearly seen in AFM images (Fig. 2a, b) and SEM images (Fig. 3a, b).

The production of exopolysaccharides (EPS) was visualized at 48 hr (Fig. 4). EPS producing capacity of *T. asahii* was 26 (µg of polysaccharide ml⁻¹ expressed per 10⁸ CFU). The extensive exopolysaccharide formation protects the microbial cells from environmental chemical toxicity (Chandran and Das, 2011). It was possible to establish thick and mucilaginous yeast biofilm on gravels after two weeks of incubation in CLM medium.

The caffeine degrading efficiencies of *T. asahii* cells immobilized in sodium alginate and *T. asahii* biofilm on gravels were studied at different initial caffeine concentration ranging from 2 to 12 g l⁻¹. The caffeine degradation increased with increasing caffeine concentration from 2 to 10 g l⁻¹ and there was a drastic decrease in the degradation percentage at 12 g l⁻¹ initial caffeine concentration. Sodium alginate immobilized *T. asahii* cells and *T. asahii* biofilm on gravels showed maximum of 80 and 89 % caffeine degradation respectively when the initial caffeine concentration was 10 g l⁻¹.

The physico-chemical parameters such as pH, COD, BOD, TDS and TSS of the effluent were 6.5, 16,700, 760, 2100 and 860 mg l⁻¹ respectively. The initial concentration of caffeine present in the effluent was found to be 11.4 g l⁻¹.

Caffeine degradation by alginate immobilized *T. asahii* was found to be 75 % and biofilm formed by *T. asahii* on gravels showed 80 % caffeine degradation over a period

**Fig. 3** : SEM images (x 3500) showing sequential biofilm formation by *T. asahii* on PVC strips during (a) 18 hr and (b) 24 hr

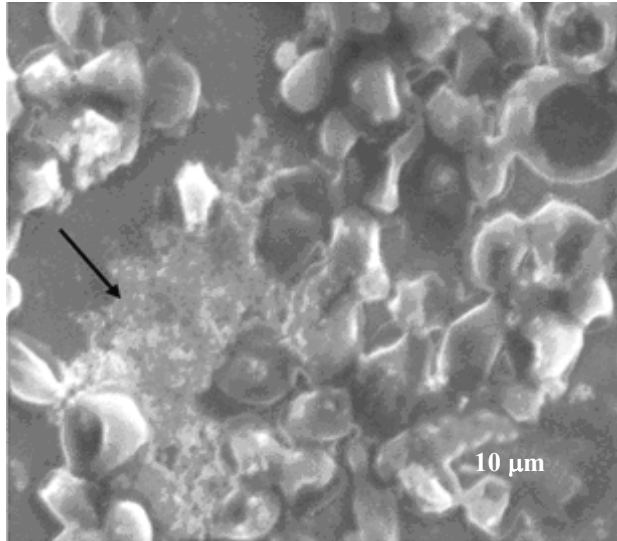


Fig. 4 : SEM images (x 5000) showing production of extrapolymeric substances by the yeast biofilm on PVC strips at 48 hr

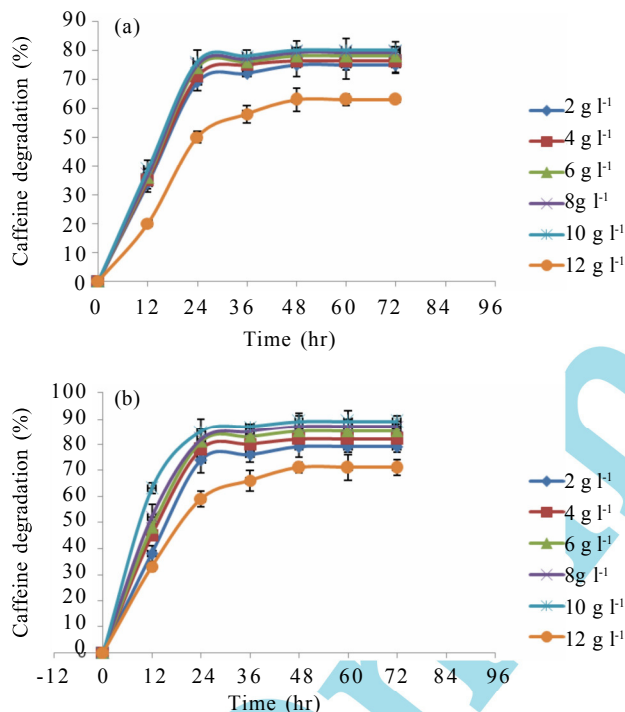


Fig. 5 : Effect of initial caffeine concentration on degradation by sodium alginate immobilized (a) *T. asahii* and (b) *T. asahii* biofilm on gravels

of 48 hr in batch mode. Thus, the present study confirmed that biofilm systems are more efficient for the degradation of caffeine. The high cell concentrations that could be achieved in biofilm systems in combination with high volumetric flow rates could potentially result in high biodegradation rate without the risk of cell washout (Heffernan *et al.*, 2009).

Further work was carried out to confirm the suitability of yeast biofilm formed on gravels for removal of caffeine from industrial effluent in a column mode. The results showed that the degradation of caffeine in the effluent was faster in the initial stages and the total reaction time was 48 hrs for the degradation up to 95 %. The SEM images of *T. asahii* biofilm on gravels before and after effluent treatment (Fig. 6 a,b) presume the survival and subsequent proliferation of the *T. asahii* cells in the biofilm matrix that enhanced the caffeine degradation. Further, the *T. asahii* cells were embedded in EPS matrix which could protect microorganisms against detrimental environment (Tolker-Nielsen and Molin, 2000; Borucki *et al.*, 2003).

Therefore, the present study showed that yeast biofilm formed on gravels presents a cost effective technique for caffeine removal from industrial wastewater. Maximum caffeine removal was noted when 'artificially' formed yeast biofilms were packed in the column which could efficiently degrade 95 % of the caffeine present in the effluent after 48 hr. Thus, it may be concluded that yeast biofilms can play

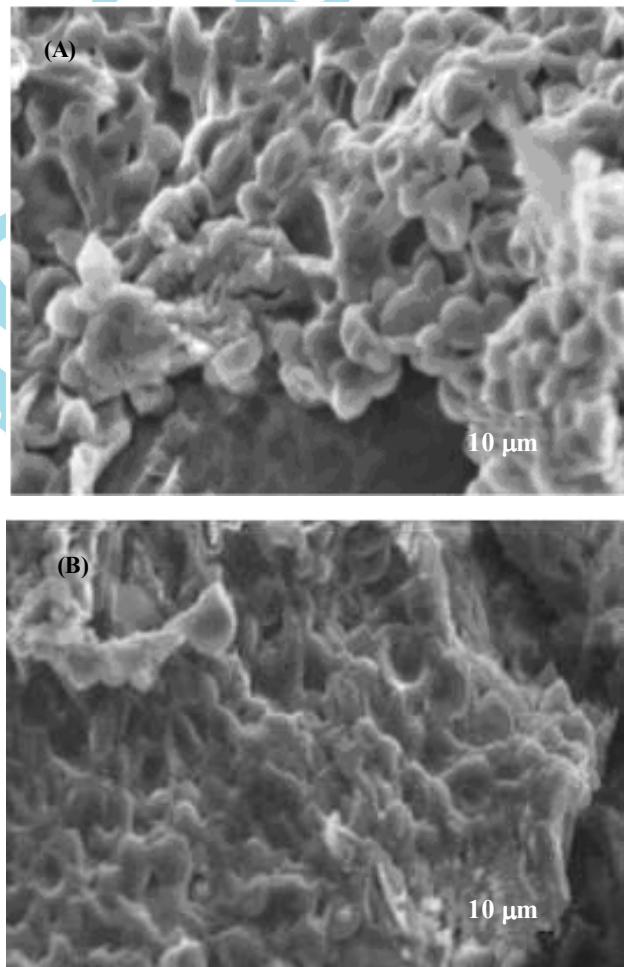


Fig. 6 : SEM images (x 5000) of *T. asahii* biofilm formed on gravels (a) before and (b) after effluent treatment

an important role in the development of cost effective biodecaffeination process for the treatment of coffee processing industrial effluent.

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