

Degradation of cefdinir from pharmaceutical waste water using immobilized *Candida* sp. SMN04 and biofilm formed on gravels

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ABSTRACT

Cefdinir being a semi-synthetic third generation cephalosporin antibiotic is considered as an emerging pollutant which demands removal from environment. Degradation of cefdinir by yeast *Candida* sp. SMN04 immobilized on various single and hybrid matrices was investigated using entrapment method. The biofilm forming ability of *Candida* sp. was evaluated by crystal violet staining assay and the formed biofilm was monitored by SEM and AFM analysis. The amount of exopolysaccharides (EPS) produced by *Candida* sp. was quantified and characterized by FTIR, HPLC and TGA analysis respectively. Cefdinir degradation from pharmaceutical wastewater was found to be 96.6% and 92.2% by PVA-alginate immobilized yeast and yeast biofilm formed on gravels over a period of 48 h in batch mode. Effectiveness of the process was also tested involving continuous-flow column studies. This is the first successful attempt on cefdinir degradation using immobilized yeast cells and yeast biofilm on solid substrate.

INTRODUCTION

Among all the pharmaceutical compounds, antibiotics and drugs are considered to be the most significant because of its high usage and consumption in both veterinary and human medicine (Benitez *et al.*, 2011). Several types of pharmaceutical residues were reported to exist in various aqueous matrices, which have received a lot of attention due to their ubiquity and adverse health effects (Arriaga *et al.*, 2009; Behera *et al.*, 2011). Among those residuals, cefdinir, a semi-synthetic third generation cephalosporin antibiotic is of great concern because of its extensive usage for the treatment of acute respiratory related disorders and mild skin infections in adults and children. Removal of cephalosporin antibiotics becomes an inevitable issue to preserve the environment as it contribute to increase the toxic strength of the effluent and production of third-generation cephalosporin resistant microorganisms (Duan, 2009; Endimiani *et al.*, 2012). Several physical and chemical techniques have been proposed in the last decades to remove antibiotic compounds from wastewater, which are of limited applicability due to inefficiency of remediating high strength wastewater, high

operating cost, huge labor requirement, high equipment cost, intervention of toxic by-products etc. (Homem and Santos, 2011). Hence, biological treatment is universally preferred because it is economical, environmental friendly and can offer the possibility of complete mineralization (Liu *et al.*, 2009). There are reports on microorganisms viz. *Pseudomonas putida* and *Pseudomonas fluorescens* (Krishnan *et al.*, 2012), *Bacillus* and *Bacteriodes* (Wagner *et al.*, 2011) and *Ustilago* sp. SMN03 (Selvi and Das, 2014a) which are capable of using cephalosporin derivatives as the sole source of carbon and energy. But free microbial cells have certain limitations viz., small particle size, possible clogging and low mechanical strength of the biomass (Godjevargova *et al.*, 2004). Microbial cells entrapped in suitable matrix have been shown to have improved tolerance to a variety of toxic and recalcitrant compounds (Chen *et al.*, 2008; Sarma and Pakshirajan, 2011). Immobilization of microbial cells has received increasing interest in the field of wastewater treatment (Tong *et al.*, 2013; Wang *et al.*, 2012). The advantages of immobilized cells include increased degradation rate, toxicity tolerance, reusability, good operational stability and low processing costs (Kadakol *et al.*, 2011; Magrí *et al.*, 2012; Wang *et al.*, 2012).

Biofilm is a highly rigid structure developed by aggregation of the microbial cells, enclosed within a matrix of exopolysaccharides (EPS) onto any inert solid material.

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They are resistant to a variety of environmental problems. EPS are microbial exudates consisting of a range of structural homopolymers and branched heteropolymers that carry out many functions such as structural integrity, cell-surface adherence and protection against environmental stress (Stoodley *et al.*, 2002). Biofilm-based processes are useful for wastewater treatment because of their simplicity, reliability, easy usage and stability (Whiteley and Lee, 2015). *Candida* sp. SMN04 isolated from pharmaceutical wastewater has already been reported which is capable of using cefdinir as sole carbon and energy source (Selvi and Das, 2014b). So far, no report is available on cefdinir degradation from pharmaceutical wastewater using immobilized yeast and yeast biofilm. Hence, the objectives of the present study are (i) to investigate the cefdinir degradation efficiency of *Candida* sp. SMN04 immobilized in single and hybrid matrices, (ii) to study the biofilm forming capacity of *Candida* sp. SMN04, (iii) to quantify and characterize the exopolysaccharides (EPS) produced by *Candida* sp. SMN04 and (iv) to investigate the cefdinir degradation from pharmaceutical waste water using immobilized PVA-alginate beads and biofilm formed on gravels in batch and column mode.

MATERIALS AND METHODS

Yeast and growth conditions

The yeast species, *Candida* sp. SMN04 (KF963314.1) isolated from pharmaceutical wastewater was used in this work (Selvi and Das, 2014b). The cefdinir degrading yeast was maintained on yeast extract peptone dextrose (YEPD) slants supplemented with 100 mg L⁻¹ of cefdinir.

Pharmaceutical wastewater

Pharmaceutical wastewater was collected from cephalosporin production unit, Chennai, 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 cefdinir concentration in the waste water was measured using UV-visible spectrophotometer.

Immobilization of yeast

The preparation of beads using immobilization matrices viz. sodium alginate, CMC, chitosan and agar was carried out under sterilized condition. Sodium alginate and yeast suspension mixture was gently dropped into CaCl₂ solution (0.2 M) to form alginate beads. The mechanical stability of the beads are enhanced by curing the immobilized beads in 0.2 M CaCl₂ solution for 2 h. Immobilized beads of yeast suspension and CMC solution mixture was prepared by gently dropping the mixture into 0.05M FeCl₃ solution and beads were further cured in the same solution for 1 h. Chitosan solution was prepared in 100 ml of 1% acetic acid and thoroughly mixed with yeast suspension. Then the mixture was gently dropped into 8% NaOH solution for the formation of

chitosan immobilized beads. The immobilized beads were separated from the solution after 30 min and washed twice with 200 mL sterile distilled water for 15 min. Agar solution (2%) was prepared under sterilized condition and melted by heating. Yeasts suspension was mixed with 100mL of the agar solution (cooled to temperature ≤ 40 °C) under sterilized condition to achieve a cell concentration above 4 g wet weight L⁻¹. Mixture of agar and yeast suspension was gently poured into sterile petri plates coated with a thin layer of refined sunflower oil and allowed to solidify for 30min at 4 °C. The solidified immobilized agar block was cut into equal size cubes (~2 mm). For the preparation of hybrid matrix, PVA (10 g L⁻¹) and sodium alginate (10 g L⁻¹) were mixed in distilled water at 80 °C. The solution was cooled and mixed thoroughly to obtain a homogenous suspension. Aliquot of 5 mL yeast suspension was added to it. The final matrix ratio was maintained as 1:1. The PVA-alginate mixtures were extruded in 0.2 M CaCl₂ to form beads of diameter 0.26 ± 0.2 cm. The resulting beads were washed with saline and used for experiments. Similar methodology was followed for preparation of glycerol-alginate beads and the same ratio (1:1) as mentioned above was maintained for the experiments.

Screening of different matrices for cefdinir degradation

To screen the best matrix for immobilization and cefdinir degradation, *Candida* sp. SMN04 immobilized on various matrices were transferred into 50 mL of YEPD medium containing cefdinir (300 mg L⁻¹) and incubated at 28 °C on a rotary shaker at 120 rpm. The samples were collected at regular time intervals to monitor degradation. The above procedure was also followed for studying the biodegradation of cefdinir by free cells by inoculating free cells of *Candida* sp. to the YEPD medium containing cefdinir (300 mg L⁻¹) and incubated under same conditions stated above.

Analytical methods for cefdinir degradation

Culture broth containing immobilized yeast was centrifuged at 8400 xg for 10 min and the supernatant was collected at regular time intervals for estimation of residual cefdinir concentration. Cefdinir concentration was analyzed by UV-Visible spectrophotometer (Hitachi U-2800) following the method Cabri *et al.* (2006). The absorbance was measured at 285 nm. The percentage of cefdinir degradation was calculated as follows,

$$\text{Cefdinir degradation efficiency (\%)} = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

where, C_i is the initial cefdinir concentration and C_f is the final cefdinir concentration.

Pellets were dried in a hot air oven at 60 °C and the dry weight of immobilized cells was measured. The dry weight of the beads without yeast cells was used as abiotic control.

Biofilm formation and quantification

Crystal violet (CV) staining assay was done to assess the cell attachment and formation of biofilms in a 96-well microtiter plate following the method of O'Toole and Kolter (1998) with

minor modifications. Microtiter plates were inoculated by adding 150 μL of inoculum grown at 24 and 48 h and incubated at 28 $^{\circ}\text{C}$. After incubation, the liquid supernatants were carefully removed using a micropipette and wells were rinsed with 180 μL of phosphate buffered saline (PBS). The attached yeast biomass was stained with 180 μL of filtered 0.1% CV solution for 10 min. The wells were then rinsed thrice with 200 μL of PBS and were air-dried. The remaining CV stain was solubilized by adding 180 μL of absolute ethanol and the light absorbance was measured at 570 nm with a Model 680 Microplate Reader (Bio-Rad laboratories Ltd, UK). Biofilm formation was scored based on absorbance value of an inoculum-free medium (control) and the biofilm. The ratio of $A_{\text{biofilm}}/A_{\text{control}}$ gives the biofilm score.

The biofilm formation was monitored using atomic force microscopy (AFM) (Nanosurf, Switzerland; Model: easyScan2) following the procedure of Lal *et al.* (2010) and scanning electron microscopy (SEM) (EVO series-MA15 SEM) analysis following the procedure of Hawser and Douglas (1994) with minor modifications.

Quantification and characterization of exopolysaccharides (EPS)

Basal minimal media containing 4% glucose, 0.3 NaNO_3 , 0.03 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 KH_2PO_4 , 0.1% yeast extract (pH 6.0) was used for EPS production (Sajna *et al.* 2013). The culture conditions were maintained for 6 days at 30 $^{\circ}\text{C}$ with 120 rpm agitation. Cell growth and EPS yield of the culture broth were studied at regular time intervals. Cell growth was determined by taking an aliquot of the medium, centrifuging it at 8400 $\times g$ for 10 min at 4 $^{\circ}\text{C}$ and drying the pellet at 60 $^{\circ}\text{C}$ for 48 h and weighed. To determine the EPS yield, the cell-free culture broth was precipitated by mixing the supernatant with twice the volume of chilled ethanol and incubated at 4 $^{\circ}\text{C}$ for 4 h. The obtained EPS was centrifuged at 8400 $\times g$ for 10 min at 4 $^{\circ}\text{C}$. The precipitated EPS was washed twice with deionized water to remove the impurities and dried at 60 $^{\circ}\text{C}$ for 48 h.

Purified EPS was characterized using an IR-affinity-1-FT-IR spectrophotometer (Shimadzu) using KBr disc, scanned in the range of 4000 to 500 cm^{-1} . The monomeric carbohydrate composition in the EPS was determined after hydrolysis with 2M H_2SO_4 at 105 $^{\circ}\text{C}$ for 3 h. The hydrolysate was then neutralized with calcium carbonate and filtered through 0.22 μm syringe filter. The monomers were determined by HPLC (Shimadzu) using carbohydrate analysis column. Deionized water was used as mobile phase and a flow rate of 0.6 mL min^{-1} was maintained.

The thermal behavior and the corresponding weight loss of the EPS produced by *Candida sp.* SMN04 was determined by the TGA analysis using thermal analyzer (DTG-60, Shimadzu, Japan). The sample was subjected to a temperature range of 0 to 700 $^{\circ}\text{C}$ under nitrogen atmosphere at a heating rate of 10 $^{\circ}\text{C min}^{-1}$.

Formation of biofilm on gravels

Biofilm was grown on gravels in YEPD medium for a period of 10 days. After incubation, the biofilm formed on gravels

were washed with phosphate buffer to get rid of loosely attached cells. The washed gravels with the biofilm was then immersed in minimal media containing cefdinir 300 mg L^{-1} for 6 days to monitor cefdinir degradation.

Degradation of cefdinir present in pharmaceutical wastewater

Experiments on degradation of cefdinir present in pharmaceutical wastewater were conducted in batch mode using PVA-alginate immobilized yeast and yeast biofilm formed on gravels by *Candida sp.* SMN04. The gravels coated with yeast biofilm and PVA-alginate immobilized yeast cells, each of 5 g were immersed in 100 mL of pharmaceutical wastewater in two separate 250 mL Erlenmeyer flasks. All the experiments were performed in triplicates under optimal conditions of pH 6.0 and 30 $^{\circ}\text{C}$. The cefdinir degradation was monitored at regular time intervals and data presented are the mean of triplicates.

In order to demonstrate the practical application of operating conditions during the degradation process in the present study, column experiments were conducted using cefdinir containing pharmaceutical wastewater. The wastewater was fed through a glass column packed with PVA-alginate immobilized yeasts at a bed height of 12 cm and flow rate of 1.0 ml min^{-1} controlled by peristaltic pump (RivotekTM).

The samples collected at regular intervals from the exit were analysed for cefdinir degradation. Similar procedure was followed for the treatment of pharmaceutical wastewater in column packed with gravels coated with biofilm formed by *Candida sp.* SMN04.

RESULTS AND DISCUSSION

Development of immobilized cells and viability

The yeast species, *Candida sp.* SMN04 was immobilized in various single and hybrid matrices viz., sodium alginate, CMC, chitosan, agar, PVA-alginate and glycerol-alginate. The cell viability was checked over a period of 12 weeks as the stability of the beads was found to play a major role in conversion of substrate to products (Idris and Suzana, 2006). From Fig. 1, it can be seen that, the yeast cells immobilized with PVA-alginate showed more stability and retained maximum cell viability than the other tested matrices.

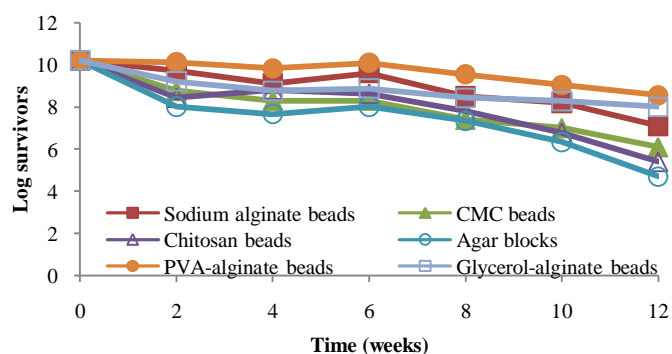


Fig. 1: Time course of changes in the viability of *Candida sp.* SMN04 immobilized in various single and hybrid matrices.

Among synthetic carriers, PVA is most preferred because it is non-toxic, non- carcinogenic (Zhang *et al.*, 2007). It also has relatively good tensile stress, impact strength, high water affinity, wear resistance, good biocompatibility, processability, minimal cell and protein adhesion and excellent electrical insulation properties (Yujian *et al.*, 2006; Zhang *et al.*, 2007). In case of PVA-alginate hybrid matrix, PVA contributes strength and high crosslinking capacity whereas alginate reduces the agglomeration and increases the surface properties (Sasaki *et al.*, 2007). PVA-alginate as a promising carrier to immobilize microbes has been reported by various workers (Yujian *et al.*, 2006; Zhang *et al.*, 2007; El-Naas *et al.*, 2013).

Table 1: Cefdinir degradation potential of free cells, immobilized yeast cells and biofilm grown on gravels grown in MB containing cefdinir at an initial concentration of 300 mg L⁻¹.

Inoculum/Immobilized matrix	Cefdinir degradation (%)
Abiotic control	0.01
Free yeast cells	84.0±0.4
Sodium alginate beads	85.6±1.8
CMC beads	84.4±0.6
Chitosan beads	81.7±1.1
Agar	79.2±1.3
PVA-alginate	88.5±1.6
Glycerol alginate	85.8±1.2
Biofilm grown on gravels	85.4±1.0

Values are mean of replicate ± SD

Cefdinir degradation by immobilized *Candida* sp. SMN04 in various matrices were performed in batch cultures and are presented in Table 1. The obtained results showed that the yeast species immobilized in PVA-alginate showed maximum cefdinir degradation over a period of 6 days compared to free cells. In a previous study, Selvi and Das (2014b) reported 84% of cefdinir degradation using free cells of yeast, *Candida* sp. SMN04 with an initial concentration of 250 mg L⁻¹ at the end of 6 days. Whereas, in the present study, PVA-alginate immobilized yeast degraded 88.5±1.6% with an initial concentration of 300 mg L⁻¹ of cefdinir within the same period of time confirming the potentiality of PVA-alginate immobilized yeast cells to degrade cefdinir more effectively than the free cells.

Biofilm formation and quantification

The adherence and subsequent biofilm formed by the yeast isolate, *Candida* sp. SMN04 was studied using crystal violet (CV) staining assay. The biofilm score of the yeast was calculated as 9.2. From the obtained results, it was evident that *Candida* sp. SMN04 was capable of single-species biofilm formation. The sequence of biofilm formation at 24 and 48h were monitored by AFM images (Fig. 2a,b) and SEM images (Fig. 2c,d) respectively.

Exopolysaccharides (EPS) production and characterization

The EPS production protects the microbial cells from environmental chemical toxicity (Chandran and Das, 2011). The total exopolysaccharides (EPS) producing capacity of *Candida* sp. SMN04 was found to be 0.29mg mL⁻¹ (expressed per 10⁸ CFU). The results showed that, *Candida* sp. SMN04 could establish a thick and mucilageneous yeast biofilm on gravels after two weeks

of incubation in cefdinir containing medium. For characterization, FTIR spectrum of the EPS produced by *Candida* sp. SMN04 was analysed and shown in Fig. 3(a). The adsorption bands obtained revealed typical polymeric structure of carbohydrates. Sharp peaks at 3259.70 cm⁻¹ and 3201.83 cm⁻¹ indicated the stretching vibration of O-H groups of carbohydrate. This characteristic group was responsible for the water solubility of EPS (Karbowski *et al.*, 2011). Two adsorption bands at 2922.16 and 2852.12 cm⁻¹ represented the C-H stretching of the methyl and methylene groups. The stretching vibration of amide group was found in the region of 1656.78 cm⁻¹ (Fanet *et al.*, 2011). Similarly, peak at 1427.32 cm⁻¹ could be assigned to C-O band of carboxylic group (-COO) (Wang *et al.*, 2010). Presence of band at 1074.35 cm⁻¹ suggested the monosaccharide constituents of pectic and hemicellulosic polysaccharides. The adsorption band at 977.91 cm⁻¹ suggested the presence of sugar monomers such as glucose, galactose and mannose in the EPS produced by *Candida* sp. On the contrary, absence of band at 870-890 cm⁻¹ indicated that β-glycosidic linkage was not present in the EPS (Tao *et al.*, 2008). Therefore, FTIR analysis of the EPS produced by yeast strain showed characteristic bands for EPS. TGA measurement is used to study the thermal stability of the materials which measures the change of sample mass (weight loss associated with dehydration, decomposition and oxidation of sample) as a function of temperature (Ahmed *et al.*, 2013). TGA of the EPS extracted from *Candida* sp. SMN04 is shown in Fig. 3(b). The plot of TGA curve is the conversion of TGA signal to percent weight loss against the sample temperature °C. Thermogram of EPS revealed that the loss of weight occurred initially (~4%) in the temperature between 22°C to 100°C which may be associated with the loss of moisture content. The moisture content of the EPS is due to the presence of high level of carboxyl group which is bound to water molecule (Kumar *et al.*, 2004) and alcohol content (Ismail and Nampoothiri, 2010). This slow decomposition of the EPS can be attributed to the presence of some thermostable saccharide moiety (Sajna *et al.*, 2013). The onset of decomposition occurred with weight loss of approximately ~12% at 234 °C, which indicates that the EPS sample was 88% stable until 234 °C. However, the compound was found to decompose to a total weight loss of ~ 65% when heated at higher temperatures upto 700 °C. The results confirmed the thermostability nature of the produced EPS at higher temperatures. Similar results were reported by other workers (Taveres *et al.*, 2005; Poli *et al.*, 2010). Thermostability of EPS is an important characteristic considered for food industry as the manufacturing and processing of several food preparations are carried out at higher temperatures. Hence, EPS produced by *Candida* sp. SMN04 being thermostable can serve as an ideal *Candidate* for food industry. Sugar analysis by HPLC of *Candida* sp. EPS revealed the presence of peak corresponding to heteropolysaccharides of glucose, galactose and mannose as shown in Fig. 3(c). The quality and quantity of the EPS depends on the carbon source and culture conditions (Laws *et al.*, 2001). The obtained result was in consensus with the contents of EPS produced by *Pseudozyma* sp. (Sajna *et al.*, 2013).

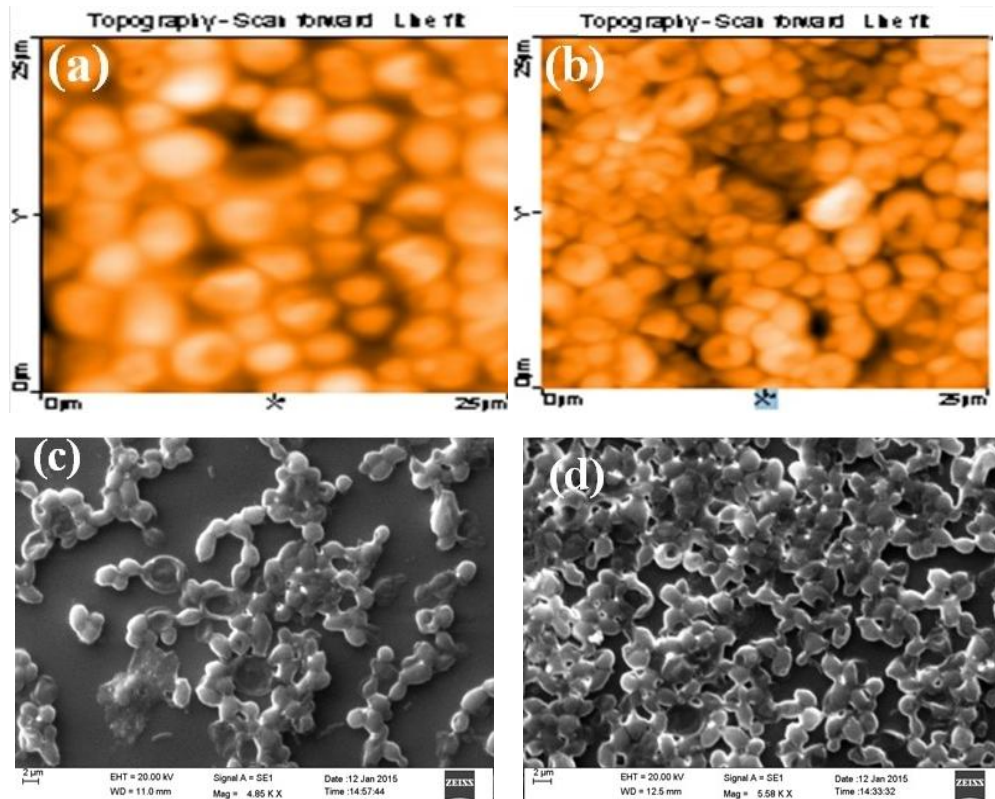


Fig. 2: AFM and SEM micrographs showing sequential biofilm formation by *Candida* sp. SMN04 at (a) 24 h and (b) 48 h.

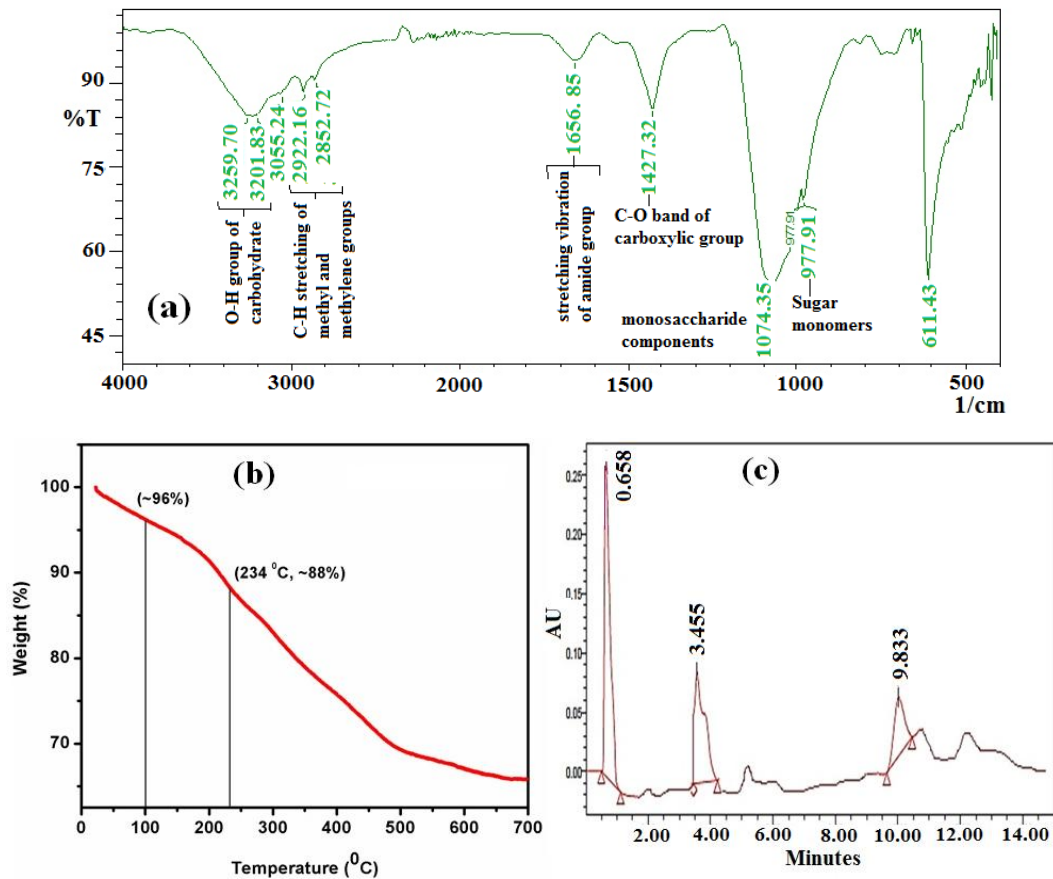


Fig. 3: Characterization of EPS produced by *Candida* sp. SMN04 EPS (a) FTIR spectrum (b) Thermogravimetric analysis (TGA), and (c) HPLC analysis of acid hydrolyzed exopolysaccharides (EPS).

Degradation of cefdinir present in pharmaceutical wastewater

The pharmaceutical wastewater was analyzed for various physico-chemical parameters such as, color, pH, COD, BOD, TDS, TSS etc following standard procedures. The initial concentration of cefdinir present in the effluent was found to be 175 mg L⁻¹ (Selvi and Das, 2014a).

Experiments were conducted to confirm the suitability of PVA-alginate-immobilized yeast and biofilm formed on gravels towards removal of cefdinir from pharmaceutical wastewater. In batch mode, cefdinir degradation by PVA-alginate immobilized yeast cells and biofilm showed 96.6±0.8% and 92.2±0.5% over a period of 48 h. Whereas in column mode, PVA-alginate immobilized yeast showed 93.5±1.1% and yeast biofilm showed 90.4±0.9% of cefdinir degradation over a period of 48 h (Fig. 4). Packed bed column studies showed that the cefdinir degradation in the pharmaceutical effluent was faster in the initial stages and it proceeded in a slower pace as the time increased. The total reaction time was 48 h, which was very much lower compared to batch studies. This may be due to the presence of cefdinir at low concentration (175 mg L⁻¹) in pharmaceutical wastewater. *Candida* sp. SMN04 has already been reported to degrade 250 mg L⁻¹ of cefdinir in aqueous medium (Selvi and Das, 2014b).

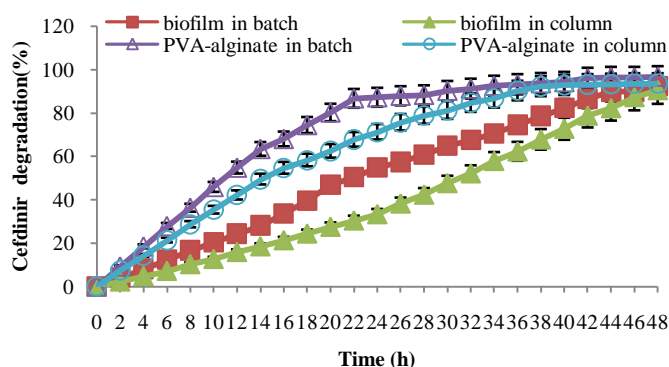


Fig. 4: Degradation of cefdinir in pharmaceutical wastewater using yeast biofilm and PVA-alginate immobilized *Candida* sp. SMN04 in batch and column mode. The data presented are the mean of triplicates.

Thus, it can be inferred that, PVA-alginate immobilized yeast cells showed higher cefdinir degradation efficiency compared to yeast biofilm. This may be due to PVA, one of the most preferred matrix for cell immobilization in various waste water treatment because of the allowance of oxygen and substrate to diffuse inside the pores to make the biodegradation process more efficient (Cheng *et al.*, 2012).

CONCLUSIONS

The results of the present study showed that PVA-alginate immobilized yeast cells are more efficient compared to yeast biofilm towards degradation of cefdinir from aqueous environment. Therefore, application of immobilized yeast may serve as an effective remediation tool for the treatment of pharmaceutical wastewater containing cephalosporin antibiotics.

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