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Original Article

ISOLATION, SCREENING AND IDENTIFICATION OF CEFDINIR DEGRADING YEASTS FOR THE TREATMENT OF PHARMACEUTICAL WASTEWATER

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ABSTRACT

Objective: The aim of the present study was to isolate, screen and identify the cefdinir degrading yeasts for the treatment of pharmaceutical wastewater.

Methods: The steps include isolation of yeasts from pharmaceutical wastewater, screening of yeasts for cefdinir degradation, studies on effects of pH, carbon and nitrogen sources on yeast growth, treatment of pharmaceutical wastewater using yeast.

Results: Out of five yeast isolates, four were screened for cefdinir degradation and identified by molecular techniques. Yeast isolates were found to utilize cefdinir as sole carbon and energy source at an optimum pH 6.0. Addition of extra carbon and nitrogen sources in the growth medium did not show any improvement of yeast growth. Maximum cefdinir degradation by four yeast isolates was found to be 72%, 78%, 81% and 84% respectively and significant reduction in BOD, COD, TSS and TDS was noted after treating the pharmaceutical wastewater with the yeast isolates.

Conclusion: The isolated yeasts may serve as potential remediation agents for the treatment of pharmaceutical wastewater containing cefdinir, a semi-synthetic cephalosporin derivative.

Keywords: Biodegradation, Cefdinir, Pharmaceutical wastewater, Yeasts.

INTRODUCTION

Pharmaceutical industries involved in the production of antibiotics discharge their wastes openly which contains some quantity of active compounds [1]. These industries often require appropriate microorganisms to reduce its COD load efficiently [2]. Wastewater from cephalosporin production units contain complicated components such as organic substances, soluble or colloid solid substances along with toxic non-biodegradable and bacteriostatic antibiotics which are matters of concern. The presence of these toxic antibiotics in the environment cause great harm to the human beings [3]. Cefdinir is an advanced third generation semi-synthetic cephalosporin antibiotic, characterized by a vinyl group at C-3 and a (Z)-2- (2-amino -4 thiazolyl) -2- (hydroxyimino) acetyl moiety at C-7 and is used for the treatment of acute respiratory related disorders and mild skin infections [4]. The effluents released from cephalosporin production units are reported to release harmful compounds which are resistant to biodegradation, photo-transformation and natural degradation [5]. The presence of high concentration of cephalosporin in the environment leads to very high chemical oxygen demand (COD) thus by increasing the toxic strength of the effluent [3]. The physico-chemical methods used for the removal of the antibiotic compounds have various limitations like high operating cost, huge labour, production of toxic metabolites etc. [6]. Hence, developing a low cost technology for wastewater treatment becomes necessary and bioremediation technology involving microorganisms can be a better option as a less expensive and more environmentally friendly alternative to the conventional treatment methods [7]. There are reports on the use of bacteria viz. *Pseudomonas putida* and *Pseudomonas. fluorescens*, *Bacillus and Bacteriods* for degradation of cephalosporin derivatives [8,9]. However, no report is available on cefdinir degradation by microorganisms. Yeasts have been found to be efficient in treating high strength organic wastewaters from pharmaceutical industries [2,10,11,12]. Therefore, preliminary study was conducted to isolate the yeasts from pharmaceutical wastewater and screen them based on their cefdinir degradation efficiencies. Furthermore, pharmaceutical wastewater was treated with the yeast isolates to test their efficiencies in terms of reduction of BOD, COD, TSS and TDS from wastewater.

MATERIALS AND METHODS

Sample collection

Pharmaceutical wastewater was obtained from the cephalosporin production unit, Chennai and Ranipet, India. Samples collected from the point of discharge into the environment were kept in clean sterile 5L plastic bottles, transferred immediately to the laboratory and stored in 4°C. Various physico-chemical characteristics of pharmaceutical waste water were analyzed using standard methodologies [13].

Chemicals

Cefdinir (<99% pure) was kindly donated by Orchid Pharmaceuticals, Chennai. Dimethyl sulphoxide (DMSO) procured from SRL Chemicals, India Ltd., was used to prepare a stock solution of cefdinir (10^4 mg/L). All other chemicals were of analytical grade and procured from Himedia Ltd, India and SRL chemicals, India Ltd.

Yeast isolation procedure

The yeasts were isolated by serial dilution method by culturing in yeast extract peptone dextrose agar (YEPD) medium containing (g/L): yeast extract-10 g, peptone-20 g, dextrose-20 g, agar-20 g. Streptomycin antibiotic was added to the YEPD medium in order to prevent bacterial contamination. The YEPD agar was autoclaved at 121 ºC for 15 min. After cooling to 50º C, the media was poured onto the Petri dishes with immediate swirling of the Petri dishes to ensure adequate mixing and uniform surface. 100 µL of various dilutions are plated onto YEPD agar by spread plate technique. Triplicates were prepared for each dilution and the sampled plates were incubated at 28 ± 2 °C for 48 h. After incubation, all the probable yeast colonies were observed by visual identification and confirmed by staining procedure through microscopy. The isolated yeasts were further streaked onto YEPD agar plates to obtain pure culture. The pure cultures were stored at 4 ºC and subcultured at regular intervals.

Acclimatization procedure

The isolated yeast species were grown in YEPD broth containing cefdinir (100 mg/L) for a period of 6 days and the cefdinir

acclimatized cultures were stored in the refrigerator for experimental use.

Screening and identification of cefdinir degrading activity of the isolates

The acclimatized yeast isolates were screened for their ability to degrade cefdinir based on their growth rate on minimal broth (MB) containing (g/L) ammonium sulphate-5 g, potassium dihydrogen phosphate-1 g, dipotassium hydrogen phosphate-2 g, magnesium sulphate-0.5 g, sodium chloride-0.1 g, manganese chloride-0.01 g, ferrous sulphate-0.01 g, sodium molybdate-0.01 g, at pH 7.2 ± 0.5. The prepared MB was autoclaved at 121 ºC for 15 min. After cooling, cefdinir (100 mg/L) was added into the MB and thoroughly mixed. Abiotic control flasks with the medium was prepared using the same composition excluding inoculum addition. The test flasks containing MB were inoculated with yeasts acclimatized in YEPD broth (OD_{600} = 0.1) and incubated at 28 ± 2 °C for 6 days on a rotary shaker at 120 rpm. All the experiments were carried out in triplicates. The cell growth of the inocula in the MB medium was determined by withdrawing the samples at regular intervals for the measurement of cell growth. Optical density values at 600 nm were measured and correlated to biomass production. Growth of the isolates were scored as abundant or high (+++), moderate (++), and minimal (+) depending upon the degree of turbidity. The yeast isolates with optimal growth were used for further studies (Table 1).

Table 1: Measurement of growth of yeast isolates in MB containing cefdinir (100 mg/L)

Isolates	Degree of turbidity
Isolate 1	++
Isolate 2	++
Isolate 3	+++
Isolate 4	+++
Isolate 5	+

The yeasts were identified to the species level using molecular identification methods by Polymerase Chain Reaction (PCR) [14]. The purified PCR products were characterized by partial and complete sequence analysis. A BLAST (Basic Local Alignment Search Tool) program was implied for similarity search from the database available on the Gen Bank [15]. The phylogenetic analysis was performed using CLUSTAL W (DDBJ-DNA Databank of Japan) [16]. The assembled partial and complete 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA sequences of the four isolates were deposited in GenBank under the following accession numbers *Pseudozyma sp*. SMN01 - KF922220, *Ustilago sp.* SMN02 - KF922221, *Ustilago sp. SMN03* - KF922222, *Candida sp.SMN04*- KF963314 respectively.

Effect of pH on growth of yeast isolates

The screened isolates were subjected to growth conditions at various pH ranging from 4.0- 9.0 by growing them in 25 mL of MB with cefdinir (100 mg/L). The flasks were incubated at 28 °C at 120 rpm in a rotary shaker. The cell dry weight of each isolate was measured at 600 nm at regular time intervals for a period of 6 days. The experiments were carried out in triplicates.

Biodegradation experiments

All the experiments on degradation of cefdinir were carried out in triplicates. The required quantity of the cefdinir stock was dispensed into Erlenmeyer flasks (100 mL capacity) containing sterile MB (25 mL) and were inoculated with the yeast cultures. The flasks used for inoculum development were incubated on a rotary shaker (120 rpm) at 28 ± 2 °C. Meanwhile, the flasks were removed at regular intervals for analysis of residual cefdinir concentration and the degradation efficiency. Uninoculated flasks were maintained as an abiotic control.

Estimation of cefdinir degradation efficiency

The Erlenmeyer flasks containing 100 mL of MB with different concentrations of cefdinir ranging from 50-300 mg/L were studied to calculate the cefdinir degradation efficiency. 4 % (w/v) of the isolates were inoculated in MB and the readings were estimated using UV-Visible Spectrophotometer (Shimadzu- UV- 2450) following a method with minor modifications [4] and the absorbance was measured at 285 nm. The percentage of cefdinir degradation efficiency 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.

Assimilation experiments - Carbon and Nitrogen sources

These experiments were conducted to determine the effect of carbon and nitrogen source on growth of yeasts and cefdinir degradation. Sucrose as carbon source and yeast extract as nitrogen source were used for this study. Carbon and nitrogen sources at concentrations ranging from 2-10 g/L were added to 250 mL Erlenmeyer flask containing 100 mL of minimal broth containing cefdinir of initial concentration 100 mg/L. All the flasks were inoculated with 4% (w/v) inoculum and were incubated at 28 ± 2 °C for 6 days on a rotary shaker at 120 rpm. The samples were withdrawn and tested for biomass accumulation at regular intervals. The obtained results were compared with readings of control flasks which contained MB and yeasts but no carbon or nitrogen sources. All the experiments were carried out in triplicates. The values obtained were mean of the triplicates.

Physiochemical analysis of pharmaceutical wastewater

The physico-chemical analysis of pharmaceutical wastewater were done following the standard methods [13]. The role of isolated yeasts in the reduction of various parameters such as BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand), TSS (Total Suspended Solids) and TDS (Total Dissolved Solids) in the pharmaceutical wastewater were studied before and after the treatment with yeast isolates.

Estimation of COD/ BOD/ TDS/ TSS

The pharmaceutical wastewater (20 mL) was inoculated with 4% (w/v) of yeasts. Control samples without the inoculum were also maintained simultaneously. All the experiments were carried out in triplicates. The samples were withdrawn at the end of the experiment for estimation of reduction in their values. The removal of BOD, COD, TDS and TSS were estimated following the formula mentioned below [17].

Removal efficiency % =

$$\frac{\text{Initial value of the untreated water} - \text{Final value after treatment}}{\text{Intial value of the untreated water}} \text{X } 100 \quad \ldots\ldots (2)$$

RESULTS AND DISCUSSION

Screening and Identification of yeasts degrading cefdinir

Five yeast species were isolated and screened for their ability to grow in MB containing cefdinir (100 mg/L) at pH 6.0. Four isolates were chosen based on their cell dry weight of 2.22, 2.73, 2.83 and 3.12 g/L respectively (Figure 1). These isolates were further identified as *Pseudozyma sp*. SMN01, *Ustilago sp*.SMN02, *Ustilago Ustilago sp. SMN03* and *Candida sp. SMN04* by molecular identification techniques.

Effect of pH on the growth of yeast isolates

The pH is one of the important parameters which favours the hydrolysis of β-lactam antibiotics [18]. The growth of the four yeasts isolates were studied at various pH ranging from 4.0-9.0 and the cell dry weight was found to be maximum at pH 6.0 for all the four isolates (Figure 2). The pH of the medium play an important role in determining the growth rate of the microorganisms which inturn is directly proportional to the degradation capability of the microorganisms [19]. At an optimized pH 6.0, the cell dry weight was recorded as 2.22 g/L for *Pseudozyma sp*. SMN01, 2.73 g/L for *Ustilago sp*.SMN02, 2.93 g/L for *Ustilago sp. SMN03* and 3.12 g/L for

Candida sp. SMN04 respectively. The results showed that pH played an important role in the growth of the yeast isolates in the medium containing cefdinir.

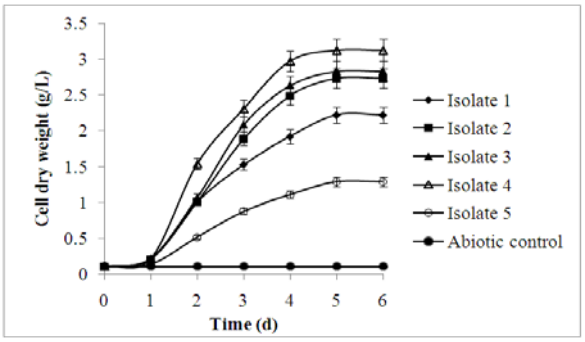

Fig. 1: Screening of yeast isolates based on their growth in minimal media containing cefdinir (100 mg/L). Data represents mean±SD

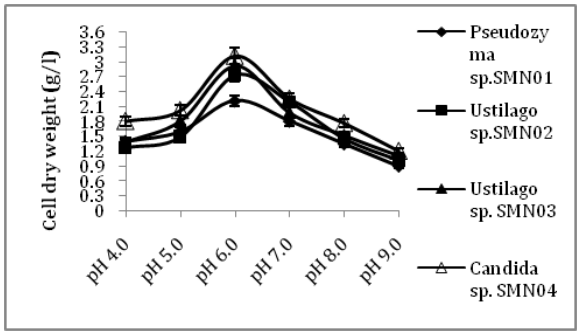

Fig. 2: Growth of the four screened yeast isolates at different pH. Data represents mean±SD

Cefdinir degradation efficiency

The four yeasts isolates were tested for cefdinir degrading efficiency by growing them in the medium containing different concentrations of cefdinir ranging from 50- 300 mg/L (Figure 3). In Figure 3, the maximum degradation efficiency was noted as 72% at 150 mg/L for *Pseudozyma sp*. SMN01, 78% at 200 mg/L for *Ustilago sp*.SMN02, 81% at 200 mg/L for *Ustilago sp. SMN03* and 84% at 250 mg/L for *Candida sp. SMN04* respectively. In a study by Hamrapurkar et al. [20], cefdinir degradation of 48.83% by base hydrolysis method was reported. There was a report on the cephalosporin degradation of concentration 175 mg/L which was removed upto 81% after 90 days of operation in a reactor [21].

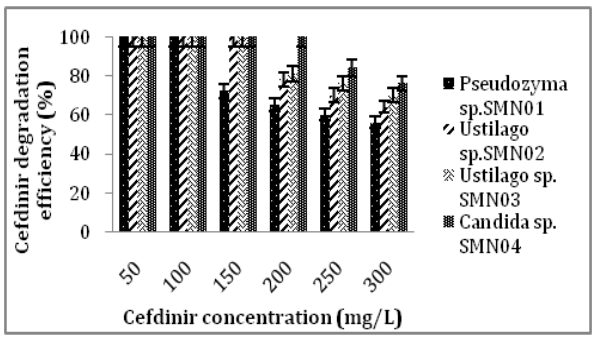

Fig. 3: Percentage degradation of four yeast isolates at various concentrations of cefdinir ranging from 50-300 mg/L. Data represent mean±SD

In another study, maximum biodegradation efficiency of cephalosporin derivatives using *Pseudomonas sp*. were reported as 64% [8]. Our study is the first report on yeasts showing the best cefdinir degrading potentiality at higher concentration. Therefore, it was noteworthy that the isolated yeast isolates of the present study could tolerate 150-250 mg/L of cefdinir and degrade 72-84% at the end of 6 days which was found to be quite high when compared with other biological and physico-chemical methods of degradation of cephalosporin derivatives reported so far.

Assimilation experiments

Assimilation experiments were conducted in minimal broth containing cefdinir supplemented with sucrose as additional carbon source (Figure 4) and yeast extract as an additional nitrogen source (Figure 5) with concentration ranging from 2-10 g/L.

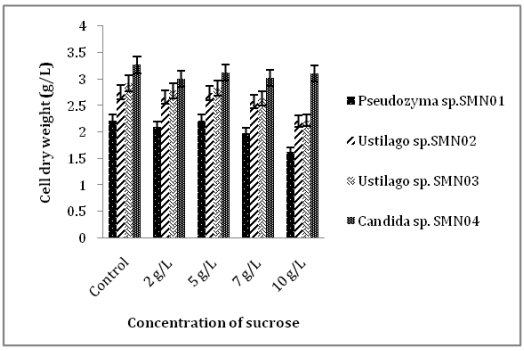

Fig. 4: Effect of various concentrations of sucrose (additional carbon source) on the growth of four yeast isolates in minimal broth containing cefdinir. Data represent mean±SD

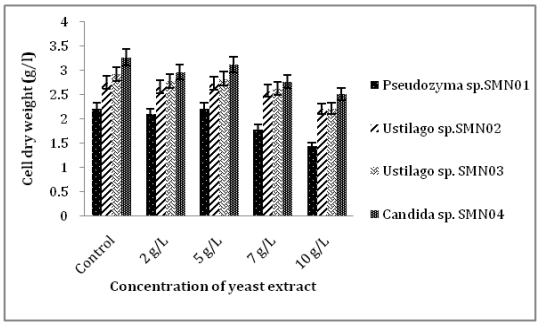

Fig. 5: Effect of various concentrations of yeast extract (additional nitrogen source) on the growth of four yeast isolates in minimal broth containing cefdinir. Data represent mean±SD

It was observed that, the addition of extra carbon and nitrogen source did not have any positive effect on the yeast's growth. Similar results were reported by other workers who reported that, the biodegradation of the antibiotics was found to amend in the presence of oxygen and absence of alternative source of carbon and nitrogen [1,22].

Physico- chemical analysis of pharmaceutical wastewater

In the present study, yeast isolates were tested for their efficiency on reduction of various parameters such as BOD, COD, TDS and TSS of pharmaceutical wastewater. Figure 6 showed that there was significant reduction of COD value when the pharmaceutical wastewater was treated with *Pseudozyma sp*. SMN01, *Ustilago sp. SMN03* and *Ustilago sp. SMN03* and *Candida sp. SMN04* compared with wastewater without yeasts. There was a report on the remediation of pharmaceutical wastewater using yeast and yeast enzymes for a period of 6 weeks, where yeasts served as efficient agents in the reduction of DO, BOD and COD of the wastewater [10].

A similar study was done by other workers [2,11] who reported that the yeasts played an important role in the reduction of COD upto 60-81% of pharmaceutical wastewater. Njoku et al [23] also reported the use of biological agents such as *Pseudomonas aeruginosa, Saccharomyces cerevesiae, and Aspergillus niger* for the reduction of COD, BOD, TSS and TDS in pharmaceutical wastewater.

Table 2 showed the reduction of various physicochemical parameters after treatment with four yeasts isolates. The reduction of COD was noted as 71.8 %, 80.4 %, 87.5% and 92.1 % in case of *Pseudozyma sp*. SMN01, *Ustilago sp*.SMN02, *Ustilago sp. SMN03* and *Candida sp. SMN04* respectively.

Out of four isolates, *Ustilago sp. SMN03* and *Candida sp. SMN04* were found to be more efficient compared to other two isolates showing TDS reduction 91.6 % and 97.7 %, BOD reduction 93.1 % and 98.7 % followed by TSS reduction 96.8 % and 100 % respectively.

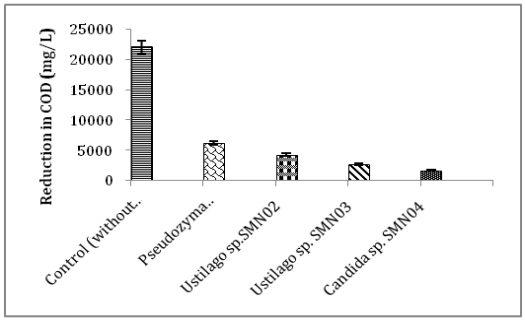

Fig. 6: Reduction in COD (mg/L) of pharmaceutical wastewater after treatment with four yeast isolates. Data represents mean of triplicates with standard deviation of <5%

Table 2: Reduction of various physico-chemical parameters of pharmaceutical wastewater after treatment with yeast isolates

Yeast isolates	TDS (mg/L)	TDS reduction (in %)	BOD (mg/L)	BOD reduction (in %)	TSS (mg/L)	TSS reduction (in %)
Permissible limits (mg/l)	3500	------	10	------	95	------
Control	19194	-------	7000	------	251	-------
Pseudozyma sp. SMN01	4069	78.8	1876	73.2	51.7	79.4
Ustilago sp. SMN02	3877	79.8	1176	84.2	25.8	89.7
Ustilago sp. SMN03	1612	91.6	483	93.1	8.1	96.8
Candida sp. SMN04	441	97.7	91	98.7	0.0	100

CONCLUSION

In the present study, yeasts were isolated from pharmaceutical wastewater and screened for cefdinir degradation efficiency. Among the four yeasts isolates, *Candida sp. SMN04* showed the maximum cefdinir degradation potentiality of 84% at 250 mg/L optimum concentration at optimum pH 6.0. All the four yeast isolates could utilize cefdinir as a sole carbon and energy source. Significant reduction in COD, BOD, TSS and TDS values were noted after treating the wastewater with the yeasts isolates. Based on the results of preliminary studies, it can be concluded that, the isolated yeasts can be effectively used for the treatment of pharmaceutical wastewater.

CONFLICT OF INTERESTS

Declared None

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