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Bacillus cereus Mediated Ɛ-caprolactam Degradation: An Initiative for Waste Water Treatment of Nylon-6 Production Plant

Mehta SK, Panchal PA, Butala BN and Sane SA*

Department of Biotechnology, Research and Development Centre, Gujarat State Fertilizers and Chemicals Limited, Vadodara-391750, India

***Corresponding author:** Sane SA, Department of Biotechnology, Research and Development center, GSFC Ltd., Vadodara 391750, India, Tel: +91 9712986358; Fax: +91 0265 2240966; E-mail: sasane@gsfcltd.com

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Abstract

Present study focuses on isolation, characterization and application of a novel bacterial strain for biological treatment of waste water from Nylon 6 production plant of Gujarat State Fertilizers and Chemicals (GSFC), Vadodara. This microbe was isolated by applying basal synthetic medium containing Ɛ-caprolactam (precursor of Nylon-6 polymer) as a sole source of carbon and nitrogen. It was identified as Bacillus cereus strain YH-10 on the basis of primary characterization followed by 16s ribosomal RNA gene analysis. This strain has very high tolerance for Ɛ-caprolactam (3.5%) compared to other reported Ɛ-caprolactam -degrading microbes. It degrades 91% and 83.3% Ɛ-caprolactam of synthetic media and Nylon-6 Waste water, respectively in 96 hrs. Ɛ-caprolactam degradation was calculated by analyzing unutilized Ɛ-caprolactam concentration in the samples using liquid chromatography-mass spectroscopy (LC-MS/MS) and chemical oxygen demand (COD) analysis. Based on these observations, biological treatment of waste water from Nylon-6 plant is envisaged utilizing minimum resources.

Keywords: Bacillus cereus; Ɛ-caprolactam-degradation; Chemical Oxygen Demand (COD) reduction; Nylon-6 waste water; LC-MS/MS

Introduction

Ɛ-caprolactam (C6H11NO) is a xenobiotic organic compound. It is an exclusive raw material for Nylon-6 polymer, which is produced by hydrolytic polymerization of Ɛ-caprolactam [1-5]. Gujarat State Fertilizers and Chemicals Ltd (GSFC), Vadodara, is a major manufacturer of Nylon-6 in India. Industrial manufacturing of Nylon-6 heavily involves Ɛ-caprolactam as an intermediate. Waste water of this plant contains un-reacted Ɛ-caprolactam as its main constituent [2] which causes significant pollution to the environment and exerts toxicity to living beings [6]. Hence this water needs treatment prior to its discharge into the environment. There is a separate treatment plant (TP-II) at GSFC which deals with processing of effluent from Nylon-6 plant. According to the environmental norms set by the Government of India, chemical oxygen demand (COD) level of final waste water should be 250 ppm or less. Reported COD value of this effluent is between 2120-7700 ppm [3]. Routinely, in order to reduce COD of this effluent measureable amount of water is added to TP-II. The objective of this study is to reduce the COD of final waste water (as per Govt. norms) through bacterial treatment. Bioremediation using selected microorganisms provide a good opportunity because it is environmentally friendly and cost effective [5]. This will remarkably reduce the quantity of water used for dilution purpose and expenditure in buying extra water.

Application of specific microorganisms for biological treatment of caprolactam containing waste waters of chemical plants can become an alternative to the existing waste water utilization methods [4]. Kulkarni and Kanekar have demonstrated applicability of Pseudomonas aeruginosa for degradation of caprolactam from Nylon-6 effluent [7].

Additionally in present scenario, major emphasis is on application of specific caprolactam tolerant microbes for waste water treatment as caprolactam composition is extremely variable in such industrial wastes. It provides better specific degradability and marked COD reduction. Several reports have shown treatment of Nylon 6 waste water utilising Ɛ-caprolactam tolerating bacteria [3]. Acinetobacter calcoaceticus isolated by Rajoo et al. [8] has shown Ɛ-caprolactam tolerance of 19 g/l and decreased the Ɛ-caprolactam content of the medium by 65% within 72 hrs (1% Ɛ-caprolactam fortified basal synthetic medium). Similarly, Sanuth et al. [9] have reported Proteus and Bordetella spp tolerating 20 g/l of Ɛ-caprolactam which exhibited 95.7% decrease in Ɛ-caprolactam concentration in medium. Baxi and Shah [3] have isolated Alcaligenes faecalis, Arthrobacter citrus, Bacillus sphaericus and Rhodococcus rhodochrous strains with Ɛ-caprolactam tolerance of 5-15 g/l. These strains have shown 95-97% decrease in Ɛ-caprolactam concentration in Nylon 6 waste water.

The current study focuses on isolation and characterization of Ɛ-caprolactam degrading bacteria from effluent of Nylon-6 plant. The work was carried out with the view of investigating the potential of isolated bacteria for Ɛ-caprolactam degradation and waste water purification. In addition, this work is an initiative to reduce the consumption of water and expenditure on waste water treatment process by GSFC.

Materials and Methods

Chemicals

Pure Ɛ-caprolactam was obtained from GSFC, Vadodara. All other chemicals and kits were obtained from HiMedia.

Collection of samples

Waste water water was collected from Vadodara Waste water Channel Limited and Waste water water of GSFC from various locations for the isolation of most effective Waste water degrading microbes. All samples were placed in separate sterile bottle and stored in a refrigerator at 4°C till use.

Isolation of Ɛ-caprolactam utilizing bacteria

Primary screening for Ɛ-caprolactam utilizing bacteria was performed by streaking a 100 µl of waste water onto nutrient agar plates fortified with different concentrations of Ɛ-caprolactam (1-4%). Plates were incubated for 48 hrs at 37°C. The fast growers were picked up and purified by sub culturing unto fresh agar plates using the streak plate technique. Since, Ɛ-caprolactam is a xenobiotic compound, it was predicted that a high concentration of Ɛ-caprolactam might be toxic to the cell growth. On the basis of Ɛ-caprolactam tolerability, GSFC-008 strain was selected for further study. To evaluate Ɛ-caprolactam tolerance of isolated strain we have recorded various parameters like colony forming units (CFU), dry weight of cells (DWC) g/l and optical density (OD). CFU of isolated bacteria was enumerated by spread plate method using 0.1 ml of the samples in 10 -1 to 10-7 dilutions onto nutrient agar. At 48 hrs the CFU of plates containing 1- 4% Ɛ-caprolactam fortified medium were counted on Remi make colony counter and calculated by following formula: CFU/ml = (Number of colonies x dilution factor) / Volume inoculated

OD of same samples was monitored turbidometrically at 600 nm on Shimadzu spectrophotometer. For percentage dry weight calculation, the samples were centrifuged at 10,000 rpm for 20 minutes on Weiber make cold centrifuge. The settled biomass was collected and dried in the oven at 100oC till it attains constant weight.

Identification of bacteria

Primary identification of selected bacteria was carried out based on its colony morphology, Gram staining and biochemical properties. Secondary identification was performed using 16S rRNA gene sequencing analysis at "BIOAXIS DNA Research Centre Pvt. Ltd", Hyderabad.

COD analysis

The isolated bacterium was tested for COD reduction property. A loopful of bacteria was inoculated in 1-4% Ɛ-caprolactam fortified basal liquid medium at 24 hrs on the shake flask level. All the samples were centrifuged for 20 mins. at 10,000 rpm and 25°C. The centrifuged supernatant was filtered through 0.45 µm filter paper. COD of this cell free supernatant was estimated by open reflux method of American Public Health Association (APHA) [1]. In this method, a sample is refluxed in strongly acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining unreduced $K_2Cr_2O_7$ is titrated with ferrous ammonium sulfate (FAS) to determine the amount of $K_2Cr_2O_7$ consumed and the oxidizable matter is calculated in terms of oxygen equivalent using the formula:

COD as mg O_2/L = [(A - B) x M x 8000] / Volume of sample (ml)

Where,

A=ml FAS used for blank,

B=ml FAS used for sample,

M=molarity of FAS, and

8000=milliequivalent weight of oxygen × 1000 ml/l.

Ɛ-Caprolactam degradation experiment

Ɛ-Caprolactam degradation study was carried out by growing bacterial strain in two experimental sets. The flask containing 1% Ɛ-caprolactam fortified basal medium was referred to as Set I. The flask containing Nylon-6 waste water (pH 7) without any supplements was referred to as Set II. 1% Ɛ-caprolactam fortified basal medium (pH 7.2) also contained KH2PO4 0.2, K2HPO4 0.6, NaCl 0.3, MgSO4.7H2O 0.2, CaCl2.2H2O 0.1, FeCl3 0.1 (g/l). Both the sets of 500 ml Erlenmeyer flasks were incubated in rotary shaker (200 rpm) at 30o C for 96 hrs. Samples from both experimental sets were collected at 0, 24, 48, 72, 96 hrs time intervals. There after the samples were centrifuged at 10,000 rpm for 30 min at 25oC, washed twice with phosphate buffer saline (PBS). These samples were tested for percentage decrease in Ɛ-caprolactam concentration by LC-MS/MS analysis (Eksigent 3200 Q TRAP) and COD reduction method.

LC-MS/MS instrument and method

LC conditions: Chromatographic study was performed using Ekspert ultraLC 100 HPLC system (Eksigent-AB Sciex, USA) coupled with 3200 QTRAP mass spectrometer (AB Sciex, USA). The autosampler system (Ekspert ultraLC 100 XL, Eksigent-AB Sciex, USA) was used for injecting 20 µL of each sample. Temperature of autosampler was maintained at 8°C while that of column oven (Ekspert ultraLC 100, Eksigent-AB Sciex, USA) was fixed at 31°C. Chromatographic elution of analyte was achieved using a Reprosil 100 C18 5 µm (150 x 4.6) mm column at a flow rate of 1 ml/min for total run time of 7 mins. The isocratic composition of eluent A and eluent B was (water with 0.1% formic acid : methanol) :: (60:40) % v/v.

MS conditions: Analysis was conducted using 3200 QTRAP mass spectrometer (AB Sciex, USA) equipped with electro spray ionization (ESI) source. The mass spectrometer was operated in the positive ion mode with a potential of 5.5 kV applied on the electro spray ionization needle. The ionization source temperature was 650°C. Ɛ-caprolactam was identified and quantified using Multiple Reaction Monitoring (MRM) mode. The curtain gas (CUR) was at 25 psi, the nebulizer source gas 1 at 50 psi and the turbo ion source gas 2 at 50 psi were utilized. The optimized declustering potential and entrance potential were 67.06 V and 7.49 V, respectively. Ɛ-caprolactam fragmentation was achived by collisionally activated dissociation (CAD) with nitrogen gas. The collision gas pressure was fixed at 2 psi for MRM quantitation. The collision energy 21 V and collision cell exit potential 4 V were optimized. Dwell time of 150 mins was used. The Ɛ-caprolactam scan was optimized by the proton aduct [M+H]+ ion at m/z 114.1 as precursor ion. The product ion at m/z ratio of 79.2 was selected.

Standard solution and calibration curve: Ɛ-caprolactam was dissolved in methanol to prepare 1 mg/ml stock solution. Working solutions in descending order of concentrations (50, 20, 10, 5 and 2.5 µg/ml) were prepared by serial dilutions using water and methanol (50:50 % v/v) with 0.1% formic acid. For the preparation of standard calibration curve of caprolactam, 100 µl aliquots of each working solution were diluted to 1 ml. This gave five concentrations (5000, 2000, 1000, 500, 250 ng/ml) of the working solution. The standard stock solutions and working stock solutions were stored at 4°C.

Sample preparation: Samples (1 ml for each set) at different time intervals were centrifuged at 10,000 rpm for 20 mins and the supernatant was filtered with 0.45 µm whatman filter assembly. These samples were diluted prior to testing with the mobile phase used in LC-MS/MS analysis.

Results

Characterization of bacteria

In primary screening the selected bacteria was identified as endospore forming gram positive aerobic rods. The colonies have a dull or frosted glass appearance with undulated margin on nutrient agar plates. Biochemical tests reveal that it utilizes citrate along with glucose and sucrose (Table 1). The given Bacterial culture was identified as Bacillus spp. JSG1 having 16S ribosomal RNA gene, partial sequence Length: 1452, Score: 2340 bits (1267), Strand=Plus/Plus. Based on the 16s Sequence data analysis, the isolated Bacterium was identified as Bacillus cereus strain YH-10 (Sequence not shown).

S.No	Characteristics		Results
	Colony morphology		Dirty white coloured, large opaque colonies, dull or frosted glass appearance with raised and undulated margin
	Gram staining and shape		Positive, Rods
	Endospore Staining and spore location		Positive and subterminal
	Motility		Positive
	Catalase test		Positive
	Indole test		Negative
	Nitrate Reduction		Positive but delayed
	VP test		Positive
	Citrate		Positive
	Methyl red		Negative
	Sugar Fermentation	Glucose	Positive
		Lactose	Negative
		Maltose	Positive
		Mannitol	Negative
		Sucrose	Positive but delayed
		Arabinose	Negative
	Polymyxin B sulphate senstivity		Resistant

Table 1: Characterization of Ɛ-caprolactam utilizing GSFC-008 isolate

Ɛ-caprolactam tolerance

The maximum Ɛ-caprolactam tolerance of the isolated bacillus strain has been found to be 3.5%. As per our knowledge this is the first report of such a high tolerance for Ɛ-caprolactam. Up till now reports of 0.5-2% Ɛ-caprolactam tolerance have been published [3,8,9]. Efficient Ɛ-caprolactam degrading potential and high concentration tolerance capacity for Ɛ-caprolactam makes our isolate an ideal candidate for clean-up of Ɛ-caprolactam contaminated environment. The isolate grown on 1% caprolactam fortified synthetic medium has shown best growth results with 53% of COD reduction in 48 hrs. With the increase in Ɛ-caprolactam concentration in medium a steep deterioration in growth has been observed. Bacteria which were grown on 3.5% Ɛ-caprolactam fortified medium have shown 97%, 74% and 80% decrease in OD, dry weight and CFU, respectively compared to growth on 1% medium. At 4% fortified medium the growth parameters are insignificant (Figure 1).

COD analysis

Originally COD of 1% Ɛ-caprolactam fortified medium and Nylon-6 waste water were 22911 ppm and 4134 ppm, respectively. When the bacteria were allowed to grow for 96 hrs, set I and set II have shown 90% and 83% COD reduction. Results of % COD reduction measured by COD analysis are in corroboration with LC-MS/MS study (Figure 2). Here it can be noted that set II has low initial Ɛ-caprolactam concentration and is devoid of any growth nutrients of basal medium. The above result is better than earlier report which has shown 80-90% COD reduction by A. faecalis in GSFC Nylon-6 Waste

water fortified with salts of basal medium [3,10]. The purpose of using unfortified Nylon-6 Waste water for this work was to reduce the Waste water treatment cost at the time of scale up at the plant level.

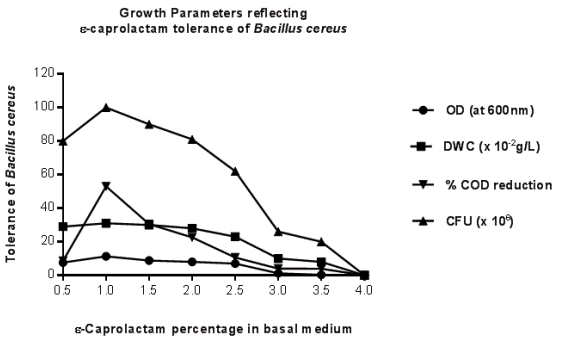

Figure 1: Ɛ-Caprolactam tolerance of Bacillus cereus on 0.5-4% Ɛ-caprolactam fortified medium. Tolerance is represented on y-axis and variable concentration of Ɛ-caprolactam fortified medium is on x-axis. Parameters monitored were % COD reduction, growth parameters like OD (at 600 nm), dry weight of cell (DWC) in g/L and colony forming units (cfu)

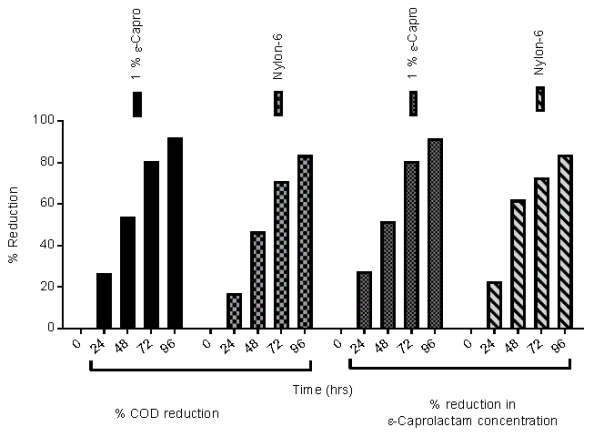

Figure 2: Bacillus cereus mediated % COD reduction and % of Ɛ-caprolactam degradation in samples incubated for 0 to 96 hrs. COD reduction was measured by open reflux method of COD analysis and Ɛ-caprolactam degradation was monitored using LC-MS/MS

Ɛ-caprolactam utilization

GSFC-008 strain which was identified as Bacillus cereus -YH-10, has shown maximum Ɛ-caprolactam tolerance (3.5%) but best COD reduction has been observed in 1% Ɛ-caprolactam group. Based on this observation further Ɛ-caprolactam utilizing experiments were performed with 1% Ɛ-caprolactam fortified medium. Bacterium was grown on basal synthetic medium containing 1% Ɛ-caprolactam as well as sterile unfortified Nylon-6 effluent.

LC-MS/MS analysis

For this purpose caprolcatm obtained from GSFC was used as standard. A standard curve was prepared using different concentrations of Ɛ-caprolactam dissolved in mobile phase (Figure 3). Q1MS spectrum of standard gave m/z values for caprolactam and its fragments (Figure 4A). A peak of 114.1g/mol molecular weight for caprolactam was obtained in product ion spectrum of standard (Figure 4B). The retention time (RT) of this peak was 3.4 mins. After 96 hrs of treatment, set I sample has shown 91% decrease in Ɛ-caprolactam content whereas in set II 83.3% bacteria mediated Ɛ-caprolactam degradation has been observed In this case, the percentage decrease in Ɛ-caprolactam contents of the samples is in correlation with their COD reduction values.

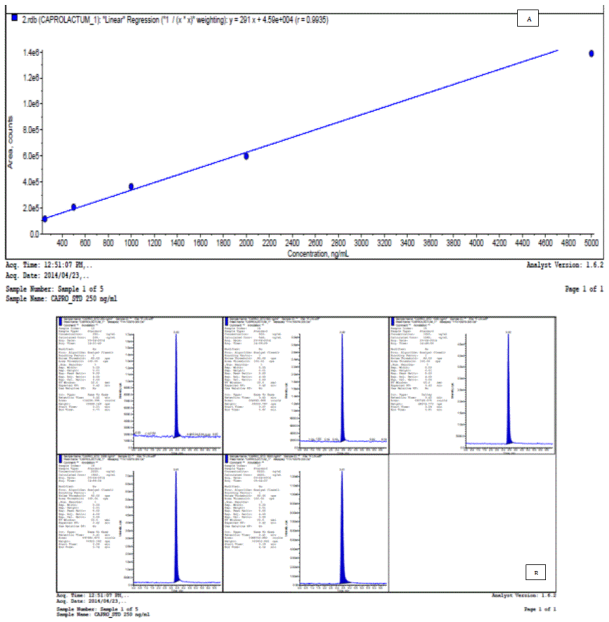

Figure 3: Calibration curve for standard caprolactam was plotted using concentrations from 250-2000 ng/ml. Retention time for caprolactam is 3.42 mins.

Growth kinetics analysis

Growth kinetics study reveals that maximum Ɛ-caprolactam degradation has been achieved during log phase of bacteria. In experimental set I and II approximately 50% decrease in Ɛ-caprolactam concentration has been noted in log phase (24-72 hrs). Same experiments showed only 11% decrease during stationary phase (72-96 hrs). OD, CFU and DWC (g/l) of bacteria in both the sets have shown two fold increase during log phase. Commencement of stationary phase reflected decline in these parameters

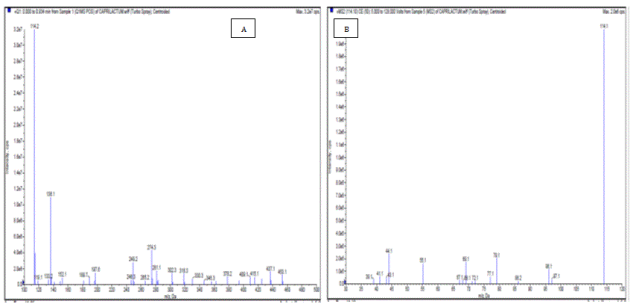

Figure 4: (A) Q1MS spectrum of standard which shows caprolactam at 114.2 m/z and other substances present in small quantity. **(B)** MS2 product ion spectrum of caprolactam and its fragments

Discussion

At GSFC, Ɛ-caprolactam is used as a monomer for the production of Nylon-6. It is often found as nonreactive component of Nylon-6 plant waste water. As per the published reports, the COD value and Ɛ-caprolactam content in this waste water is between 2120-7700 ppm and 1000-3600 ppm, respectively [3]. By means of the present work we have successfully reduced COD of Nylon-6 effluent from 4134 ppm to 700 ppm (83%). This is a milestone by which we can greatly reduce the water consumption for waste water treatment and this will also save extra cost to achieve the acceptable COD value. In addition to this, with increase in aeration conditions of effluent and strain improvement, we can achieve 94 % reduction (250 ppm) in COD value which is our ultimate goal. In near future we will be performing scale up studies in pilot plant with the bacteria based fermentor. The results obtained from pilot plant studies could be extrapolated for setting up an industrial scale up unit.

In this study, Bacillus cereus strain YH-10 exhibits maximum tolerance for Ɛ-caprolactam (3.5%) which is a first report of its kind. Such a high Ɛ-caprolactam tolerance can be attributed to spore forming ability of the bacteria. Ɛ-Caprolactam content of waste water varies considerably during adverse conditions in the plant. High caprolactam tolerance of this strain thus will be promising in treatment of Nylon-6 effluent at plant level. It also exhibits high Ɛ-caprolactam degradation efficiency by decreasing the Ɛ-caprolactam content of synthetic medium to 91% and that of Nylon-6 waste water by 83.3%.

Conclusion

Therefore, Bacillus cereus strain YH-10 isolated in this study has a better e-caprolactam utilization potential compared to those of earlier reports and will be expected to play a better role in remediation of e-caprolactam polluted Nylon-6 effluent.This will ultimately result in huge reduction in quantity of water and the cost previously required for this purpose.

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