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A novel mariner-based transposon system for the enhanced removal of high strength ammoniacal nitrogen in pharmaceutical effluent

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Abstract

Industrial wastewater is the major polluting agent in the environment as huge amount of untreated effluent is discharged from industries causing serious effect to biotic system. The pharmaceutical industry effluent used in the present study contains high concentration of ammonical nitrogen (NH_3-N) about 500 mg L^{-1}. In the present study, efficient NH_3-N removing strain was isolated, and enriched in the effluent with high NH_3-N concentration for its efficient removal. The strain which showed higher removal efficiency was identified as *Proteus penneri* by ribotypying. The wild type *P. penneri* exhibited low removal efficiency (64%) in 24 hours even after the conventional enrichment method. Hence, the strain was mutated to improve its degradation efficiency using modified mariner based transposon system. It was constructed by replacing the Kan^r gene with Gm^r gene to develop pSC189::miniTn(Gm), since the isolated strain was resistant to kanamycin. Two mutant strains T_{55} and T_{132} were shown to have enhanced NH_3-N removal efficiency by 84% and 81% respectively, in 24 hours. The kinetic rate constants such as pseudo first and second order kinetics were evaluated for the degradation of NH_3-N by wild type *P. penneri* and transposon mutant; both of them followed second order rate kinetics. The NH_3-N removal was confirmed by Ion chromatography (IC) and Fourier Transform-Infrared Spectroscopy (FT-IR). To date, there is no report on the strain improvement using transposon mutagenesis for the treatment of NH_3-N.

Keywords: NH_3-N removal; Random mutagenesis; Transposan mutagenesis; *Proteus penneri*; Pharmaceutical effluent treatment.

1. Introduction

Waste water released from various industries becomes the major concerns to environmentalists since they contain complex nature of chemicals with poor biodegradability.[1] Ammonium salts are present in certain industrial wastewaters such as leather, chemical, pharmaceutical and pesticide manufacturing industries.[2] Pharmaceutical industries all over the world generate large quantities of wastewater that contain considerable concentration of antibiotics, organic compounds as well as ammoniacal nitrogen (NH_3-N). The conventional methods such as chemical precipitation (Magnesium Ammonium Phosphate (MAP) process),[3,4] air stripping process,[5] ion exchange,[6] etc. have been examined and each process has its own demerits in field level implementation. The chemical precipitation generates huge amount of toxic sludge and it needs to be dumped in landfill sites and the landfill leachates cause major environmental problems. The air stripping process releases the toxic ammonia into the air and the ion exchange process is not economically viable since it needs to be regenerated after the removal. Thus, the biological process is preferred, since it overcomes the demerits caused by physico-chemical treatment methods. Mainly, the biological process is eco-friendly in nature, since it provides the sludge free treatment. The economic advantage, both in terms of capital investment and operating costs of biological treatment over other conventional treatment processes, has cemented its place in any integrated wastewater treatment plant. The major problem with biodegradation of pharmaceutical effluent is the presence of high concentration of NH_3-N (>100 mg L^{-1}); such high concentration impairs the biodegradation of antibiotics and other organic compounds in effluent due to the toxicity of NH_3-N exerted to the microorganisms. Therefore, in pharmaceutical effluent, it becomes important to remove the NH_3-N, inorder to ease the biodegradation of antibiotics and organic compounds by the microbial consortium.

Therefore, the focal theme of the present investigation was to isolate the ammonia utilizing bacterial strain *Proteus penneri*, enrich and use for the removal of ammonia from pharmaceutical industry effluent (PIE).

Commonly, 16S rDNA molecules are the tools to investigate microbial communities that avoid limitations of culturability. Several functional genes were to be useful for the same purpose because their phylogeny are congruent or very similar to phylogenetic relationships based on 16S rRNA gene (rDNA) analyses and moreover they indicate functional diversity in the environment.[5]

This paper mainly discusses about the biological method for the removal of NH_3-N in PIE by *P. penneri*. In addition, the study was aimed to extend the modification and incorporation of a mariner transposon in the genome of *P. penneri* to generate libraries of random insertion mutants. The mariner transposons as demonstrated by Linong *et al*.[7] was expected to move through a DNA intermediate during transposition using a 'cut-and-paste' mechanism, resulting in excision of the transposon from the original location and insertion at novel sites in the genome. Lionel *et al*.[8] reported that they always integrate into a TA target dinucleotide, which causes insertional inactivation of the particular site. The libraries are then screened to identify mutants that are defective in a particular phenotype so that it shows enhanced removal of NH_3-N in a short duration of time. This approach is cost-effective and applicable to a wide variety of bacteria.[9] These "suicide" vectors are rapidly lost but allow the integration of passenger exogenous DNA carrying selection markers through transposition or homologous recombination in the chromosomes of recipient strains.

Therefore, in the present study, evaluated the use of *Himar1 mariner* transposons[10] for random mutagenesis of *P. penneri* and the efficient bacterial strains were isolated by screening

the transposon mutants from its library. They were further enriched in the industrial effluent and their efficiency was increased to further enhance the efficient treatment in a short duration of time. To date, there is no report on the treatment of NH_3-N by the strain which is modified by the method of transposon mutagenesis.

2. Materials and Methods

2.1. Materials

The ammoniacal nitrogen containing pharmaceutical wastewater was obtained from a pharmaceutical industry, Chennai, India. The chemicals used in the study were purchased from Himedia and Sigma-Aldrich-Fluka Chemical Co., India. The primers were purchased from Bioserve, Chennai. All the enzymes used for molecular biology works were purchased from New England Biolabs (NEB), Chennai, India.

2.2. Isolation and identification of ammonia oxidizing microbial strain

The efficient ammonia oxidizing bacterial strains were isolated from the PIE contaminated soil by the enrichment method. The enrichment was started with the introduction of contaminated soil in the industrial effluent and incubated it for 96 hours under aeration (1 L min^{-1}) and the grown up bacterial cultures were used as mother inocula for the further periodic enrichment. The culture was then periodically transferred into the fresh effluents in 3 days interval where the enrichment was continued upto 3 weeks. Then the bacterial strains which acclimatized with the PIE were screened for the better efficiency in the removal of NH_3-N. Around 3 different bacterial strains were isolated from the enriched sample. Amongst 3 strains, the strain which showed higher efficiency in the removal of NH_3-N was subjected to molecular identification by ribotyping.

To identify the strain, fD1 [CGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG] and rP1 [CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT] primers[11] were used for amplification of the variable region of the 16S rDNA gene. The deduced sequence was subjected to BLAST analysis against NCBI database. Phylogenetic analysis was performed by subjecting the deduced sequence to the 16S rDNA data base to obtain the closely related sequences, and the phylogenetic tree was constructed, based on evolutionary distances that were calculated by following the distance matrix method, using the Phylip package.[12] The strain was maintained in Luria Bertani (LB) medium for the further use.

2.3. Removal of ammoniacal nitrogen in industrial effluent by wild type strain Proteus penneri

The removal of ammoniacal nitrogen in PIE was carried out in 1L batch reactor. The enriched *P. penneri* culture was centrifuged at 10,000 rpm for 10 minutes and the pellet was collected and inoculated in 1L PIE. The moisture content of the biomass is 0.2 g g^{-1} of biomass. Various parameters were optimized for the enhanced removal of NH_3-N from PIE by varying the time (24, 48, 72 and 96 hours), pH (6.0 to 9.0) and biomass (inoculums) concentrations (10, 20, 30, 40 and 50 g (wet weight) L^{-1} of effluent. The effect of trace elements on the removal of NH_3-N was determined by adding 2 ml of trace element solution per litre of PIE. Trace element composition was EDTA (5 g L^{-1}), $ZnSO_4.7H_2O$ (2.2 g L^{-1}), $CoCl_2.6H_2O$ (1.6 g L^{-1}), $MnCl_2.4H_2O$ (5.1 g L^{-1}), $CuSO_4.5H_2O$ (1.6 g L^{-1}), $(NH_4)_2Mo_7O_{24}$ (1.1 g L^{-1}), $CaCl_2.2H_2O$ (5.5 g L^{-1}) and $FeSO_4.7H_2O$ (5 g L^{-1}). In addition to the trace elements, the salts such as $(NH_4)_2SO_4$ (1 g L^{-1}), KH_2PO_4 (0.07 g L^{-1}) and $NaHCO_3$ (3 g L^{-1}) were added to improve the growth of the *P. penneri* and thus the removal efficiency of NH_3-N. Aeration was provided at the rate of 1 L min^{-1} and results were analysed for 96 hours in 24 hours interval. The removal of ammonia was analyzed by using Kjeldhal method[13] and further the nitrite and nitrate which are formed from the

conversion of NH_3-N were analyzed using the colorimetric methods followed by Narayana and Sunil[14].

2.4. Construction of transposon mutant library

A transposon mutant library of *P. penneri* was constructed by using a modified mariner transposon, pSC189::miniTn(Gm), wherein, the Kan^r gene in the transposable element was replaced by a Gm^r gene (Fig.1), since the wild strain was initially found to be sensitive to gentamycin.

A transposon mutant library of *P. penneri* was constructed by using a modified mariner transposon, pSC189::miniTn(Gm), which is developed by replacing KanR gene in pSC189 with GmR gene from pGp-Tn7-Gm. The Gmr gene was PCR amplified with the primer listed in table 1 that introduce the desired restriction sites Xho1 and RsrII for further cloning. The PCR products was digested using XhoI/RsrII restriction enzymes and ligated in pSC189 which is pre-digested with same enzyme to delete Kmr gene as illustrated in Fig. 1, to give plasmid pSC::miniTn(Gm). This resultant plasmid is preferred to creating mutant library of *P. Penneri* since the wild strain was found to be sensitive to gentamycin. The procedure was adapted from Tn*7*-based cloning and delivery system developed by Sebastien *et al*.[15] and Neal *et al*.[16] The cultures with appropriate doses of antibiotics were supplemented at the following concentrations: Gm, 15 mg ml^{-1} (w/v) and Amp, 100 mg ml^{-1} (w/v) and they were incubated at 37°C. All the primers used for vector construction were presented in Table 1. The Gm^r gene from pGP-Tn7-Gm was PCR amplified, digested using XhoI/RsrII restriction enzymes and cloned in pSC189 which is pre-digested with same enzyme to delete Km^r gene as illustrated in Fig. 1, to give plasmid pSC::miniTn(*Gm*). The positive clones were further selected on a selective media of LB supplemented with Amp_{50} and Gm_{15}.

For the conjugal transfer, pSC::miniTn(*Gm*) were further transformed to Mu-free donor (MFD) cells to act as donor, grown in LB supplemented with 0.3 mM Diaminopimilic acid (DAP). Conjugation was performed according to the procedure followed by Lionel *et al*.[8] with little modification as follows: equal volumes of the donor and recipient cells to read optical density of 1.0 at 600 nm was mixed, centrifuged to collect the pellet and washed twice with LB broth. The pellet was further suspended in 50 µl of LB broth and spotted on dry LB agar plates to make tight contact for conjugation. After incubation of plates for 1 hour at 37°C, the cell spot was harvested and resuspended in fresh LB broth. The transconjugants were further selected on an LB plate containing Gm_{15} by spread plate after overnight incubation at 37ºC. The absence of DAP provided the non selection of MFD donor strain. They were confirmed for the transposon insertion by colony PCR using R6K primers, which were amplified the R6K origin of replication located inside the transposable elements. Genomic DNA of the transconjugants was used as the template for PCR amplification.

2.5. Screening of the mutant with enhanced ammoniacal nitrogen removal

Following mutagenesis, viable mutants were screened for enhanced NH_3-N removal. The removal ability of the mutants was verified from reduction profiles of different mutant strains and compared with wild strain under the optimised conditions. For this purpose, a total of 350 isolates were screened, each of which was inoculated into 1 L of PIE, and incubated for 24 hours under optimum conditions together with the addition of other trace elements and salts. The residual NH_3-N content was determined in the treated effluent by Kjeldhal method.[13] Reduction profile of each of the mutant strain was plotted and analysed.

194 *2.6. Kinetic rate constants for the degradation of NH_3-N by P. Penneri wild type and transposan*
195 *mutants*

196 In order to investigate the kinetic rate constants for the degradation of NH_3-N using *P.*
197 *penneri*, the non-linear kinetic models were applied. The pseudo first order[17] and pseudo second
198 order[18] kinetic models were employed, following the equations, *Eq.* 1 and 2 respectively.

199 $$r_t = r_e(1 - \exp^{-K_1 t}) \quad (1)$$

200 $$r_t = \frac{K_2 r_e^2 t}{1 + K_2 r_e t} \quad (2)$$

201 where r_e and r_t are the amounts of NH_3-N (%) degraded at equilibrium and at time (t) , k_1 and k_2
202 are the first and second order rate constants.

203 *2.7. Fourier transform-infrared spectroscopy (FT-IR) studies*

204 A Perkin Elmer infrared spectrometer was used for the investigation of nitrite and nitrate
205 ions in treated PIE sample. The treated and untreated samples were lyophilized and mixed with
206 KBr and made into pellets at a pressure of about 1MPa. The pellets with dimensions diameter 10
207 mm and thickness 1 mm, the samples were scanned in the spectral range of 4000–400 cm^{-1}.

208 *2.8. Quantification of nitrite and nitrate in treated and untreated effluent by colorimetric method*
209 *and ion chromarography (IC)*

210 The nitrite and nitrate produced from the conversion of NH_3-N by wild and mutant strains
211 were analyzed using the colorimetric methods followed by Narayana and Sunil[14]. Also, the ion
212 chromatography was used to confirm the conversion of NH_3-N into nitrite and nitrate ions. The
213 ion chromatography was performed with Metrohm instrumentation consisting of a 709 IC Pump,
214 733 IC Separation Centre and a 732 IC conductivity detector. Cation separation was carried out
215 in non suppressor mode on a Metrosep cation 1-2 analytical column (125 × 4 mm) connected in

series with a Metrosep cartridge. The Metrosep cartridge is used as a precolumn to protect the analytical column. The dilution factor for each of the sample was fixed at 50. Sample injection volume used was 20 µl. The nitrite ad nitrate quantity in mg L^{-1} was determined from the peak area and time in minutes from the chromatogram.

3.0. Results and Discussion

3.1. Isolation and identification of ammonia oxidizing microbial strain

The strain with enhanced ammonia removal was isolated and identified as *Proteus penneri* by the method of ribotyping. The 16S rDNA sequencing data indicated that the isolate was *P. penneri* (Fig. 2). The nucleotide sequence reported here has been assigned an accession number "KM000848" from NCBI Gene Bank database. The BLAST result showed 99% similarities to those of nearest strain *P. penneri* NCTC 12737. NH_3-N analysis by Kjeldhal method[13] showed that the major reduction was obtained with enriched wild strain of *P. penneri* wherein it was reported to have an efficient ammonia removal by 64% in 24 hours.

3.2. Optimization of culture conditions for the removal of ammoniacal nitrogen by wild type P. penneri

3.2.1. Effect of time

The effect of time on percentage removal of ammonia by wild type strain was studied and was observed that longer the incubation time, the better the efficiency of NH_3-N removal and it was 64% in 24 hours and 74% in 72 hours (Fig. 3a). It was observed that increase in treatment period beyond 72 hours decreased the removal percentage for the wild strain. Several factors such as toxicity of the other chemicals presented in the sample or secondary metabolites produced during the treatment of PIE could be attributed to the decrease in efficiency. Tang *et al.*[19] reported that they achieved around 78% ammonium removal efficiency with the initial

effluent NH_3-N concentration 123-257 mg L^{-1} by the ammonia oxidizers in the anaerobic ammonium oxidation (ANAMMOX) process. Comparatively, *Proteus penneri* can be considered as highly efficient strain for the removal of high strength ammoniacal nitrogen containing wastewater since it showed 74% efficiency with the initial effluent NH_3-N concentration 500 mg L^{-1}. To our knowledge, such a high ammoniacal nitrogen removal capacity for pharmaceutical wastewater has not been previously reported.

3.2.2. Effect of pH

The pH of the culture is one of the most important environmental parameters affecting microbial cell growth and enzyme production. The effect of initial pH on the NH_3-N removal by *P. penneri* was investigated for pH values from pH 6 to 9 as shown in Fig. 3b. The maximum removal of NH_3-N was observed at pH 8. The oxidation of ammonia by nitrifying bacteria growing in inorganic liquid media generally occurs optimally within the pH range 7.0-8.5.[20, 21] Also, the most likely mechanism for inhibition of growth in lower pHs are ionization of NH_3 to NH_4^+ at low pH and either the lack of an active ammonium transport system.[20]

3.2.3. Effect of temperature

The temperature has greater role in the removal of NH_3-N. The maximum removal of NH_3-N (64%) was observed at 35°C. The efficiency of the NH_3-N removal was not much affected at 40°C, but at 45°C and 50°C, the removal efficiency was gradually decreased (Fig.3c). This indicates that the optimum temperature for the removal of NH_3-N by *P. penneri* was 35°C and the bacterial strain may be regarded as mesophilic in nature.

261 *3.2.4. Effect of biomass concentration*

262 The selection of suitable biomass concentration becomes an important parameter to
263 improve the NH_3-N removal efficiency since the efficiency is controlled by the number of cells.
264 Hence, various concentrations of biomass 10 to 50 g L^{-1} (w/v) were added to 1 L PIE at pH 8.0.
265 The results showed that NH_3-N removal efficiency was achieved by 64% with biomass
266 concentration of 40 g L^{-1} (w/v) and there was no much difference in NH_3-N removal efficiency
267 for increase in biomass concentrations beyond 40 g L^{-1}. This infers that 40 g L^{-1} of biomass was
268 required to provide the maximum removal of NH_3-N in 1 L of PIE (Fig.3d).

269 *3.3. Construction of pSC189::miniTn(Gm)*

270 In the present study, pSC189 was chosen to be the suitable transposon delivery vector for
271 *P. penneri*, as they are non-replicative plasmid with R6K origin of replication and hence
272 replicate only in those system which encodes the λ-pir protein[8]. The wild stain *P. penneri* was
273 observed to be sensitive to gentamycin and resistant to kanamycin, and thus more versatile
274 vector was developed in the present investigation by replacing the kanamycin resistant gene with
275 gentamycin resistant gene (Supplementary file-Fig.1). As suggested by Lionel *et al*.[8] and Chiho
276 *et al*.[22] the replication of these plasmids requires the pir-encoded protein, which is usually
277 provided in trans in the donor strain. In the absence of pir site in the recipient strain, these
278 plasmids cannot replicate.

279 For the conjugal transfer, pSC189::miniTn(*Gm*) were further transformed to MFD λ-pir
280 donor strain. After conjugation with *P. penneri*, the transconjugants were selected on LB with
281 gentamycin in the absence of DAP. The donor MFD λ-pir cells will not survive since it requires
282 the supplementation of DAP, rather transconjugants alone will survive on the gentamycin plate.

The transconjugants were confirmed by PCR amplification of R6K origin of vector backbone (supplementary file-fig.6).

3.4. Screening of the mutant with enhanced ammonia removal

About 350 mutant colonies were screened from transposon mutant library. The NH_3-N removing mutants were screened by inoculating the mutants in the PIE while the wild type strain served as control. Of which, 2 mutants (T_{55} and T_{132}) showed enhanced removal of NH_3-N compared to other mutants. NH_3-N content was estimated for the mutated strains under the optimized conditions and was found to be 84% and 81% in 24 hours for the transposon mutants T_{55} and T_{132} respectively. Since the T_{55} mutant strain showed higher efficiency when compared to T_{132}, the T_{55} was selected for the further study. The mutant T_{55} was proved to have an improved efficiency of NH_3-N removal as they showed a high removal efficiency of 84% within 24 hours and 89, 95 and 97% in 48, 72 and 96 hours respectively, whereas the wild type strain removed NH_3-N by only 64% in 24 hours and 70% in 96 hours. This indicates that, the *P. penneri* transposon mutant was an inevitable source for the enhancement of the NH_3-N removal. The stability of the transposon mutant strain, T_{55} was confirmed even after five subcultures in non-selective media.

3.5. Hydrolysis rate kinetic constants

The validity of the kinetic order of degradation process was based on the regression coefficients. The first order rate constant k_1 and the second order rate constant k_2 are summarized in Table 2. The results confirmed that the degradation of NH_3-N obeyed the second order rate kinetic model as observed greater R^2 values.

3.6. Characterization of the treated sample using FT-IR studies

The FT-IR spectrum of untreated effluent (Fig. 4a) has a wide band at 3432.71 cm^{-1}, due to the 1º and 2º amines - stretching vibrations caused due to the presence of ammonium ions present in industrial effluent. The peak observed at 2358.3 cm^{-1} is attributed to NH_2 scissoring (1º amines) – bending vibrations and the peak observed at 1083.637 cm^{-1} are due to C–N stretching vibrations.

The FT-IR spectrum of the PIE treated with wild –type strain (Fig. 4b) shows the peak at 1398.11 cm^{-1} suggest the presence of aliphatic NO groups and the peaks at 1666.71 cm^{-1} is due to the asymmetrical stretching in NO_2. The bands at 834.50 cm^{-1} and 703.48 cm^{-1} could be attributed to stretching of π bond of N-O linkage and the presence of NO_2 bending vibrations.

The spectral data of various peaks corresponding to the PIE sample treated with transposon mutant T_{55} (Fig. 4c) indicated that the peaks at 1634.43 cm^{-1} and 788.964 cm^{-1} correspond to NH_2 scissoring (1º amines) – bending vibrations and NH_2 and NH wagging respectively. The peak at 1402.134 cm^{-1} indicates the presence of aliphatic NO groups and peak at 788.964 cm^{-1} indicated NO_2 bending vibrations.

Therefore, it is observed that the intensity of the 1º and 2º amines stretching vibrations, caused due to the presence of ammonium ions, has decreased in the treated effluent when compared to the untreated sample. Hence, it can be validated that the presence of various nitro (nitrite and nitrate) groups in the treated sample indicates that the major removal of NH_3-N has occurred through the conversion of amine groups to nitro groups.

3.7. Nitrite and nitrate determination in the treated and untreated effluent by colorimetric method and ion chromatography (IC)

The treated PIE sample (24 hours sample) by wild type and mutant strains were subjected to nitrite and nitrate analysis by colorimetric methods, it shows the nitrite and nitrate production were enhanced in the treated PIE sample by the mutant strain compared to wild type strain. The treated PIE by wild type strain contains around 12.6 mg L^{-1} of the nitrite and 4.8 mg L^{-1} of the nitrate and the treated PIE by mutant strain contains around 61mg L^{-1} of the nitrite and 26 mg L^{-1} of the nitrate. This was further confirmed with ion chromatography.

Ion chromatogram was collected to determine the quantity of nitrite and nitrate ions present in the treated and untreated PIE sample (Figure not shown). The data obtained from the chromatogram validates that the quantity of nitrite present in the untreated PIE sample was 23.7 mg L^{-1} and the nitrite present in the PIE sample treated with wild type strain *P. penneri* was 26.2 mg L^{-1}. This shows that 9.5% conversion of NH_3-N to nitrite ions has occurred during the treatment process with the wild strain in 24 hours under optimized conditions. The treatment efficiency of the transposon treated sample shows a major conversion of 56.7% as nitrite present in the transposon treated effluent has increased from 23.7 mg L^{-1} to 54.8 mg L^{-1}. Around 23.6% nitrate was observed in the transposon treated sample while it is 5.2% in the wild type treated sample.

Therefore, it is inferred that major removal of NH_3-N was caused due to the conversion to nitrite ions and a minor conversion to nitrate ions. Hence, it is studied that the amoA gene which is responsible for the expression of ammonia monooxygenase, which is the first enzyme involved in the conversion of ammonia[23] to nitrite plays a key role in the process of NH_3-N removal from the effluent sample. This is corroborated with the findings of Zhi *et al.*[24] and also with Zhi and

Ji[25]. They reported that the ammoniacal nitrogen removal is collectively controlled by amoA and nxrA gene.

4. Conclusion

In this study, the *P.penneri* was isolated and enriched in the PIE for the NH_3-N removal. The wild strain showed only 64% and 74% NH_3-N removal in 24 and 72 hours respectively. The strain improvement was done for the purpose of industrial applications by reducing the reaction time and increasing the efficiency of the strain by constructing a mutagenesis library of *P. penneri* by the method of transposon mutagenesis. A series of mutant strains were developed for the purpose enhanced removal of NH_3-N in PIE. NH_3-N removing mutant strains T_{55} and T_{132} were acquired from the transposon mutation bank, which exhibited increased NH_3-N removal than the wild strain under the optimized conditions and in short duration (24 hours). The selected mutant strain T_{55} were found versatile for the removal of NH_3-N by 84% in 24 hours. Hence, random mutagenesis was found to be an efficient method for the strain improvement to enhance the removal of NH_3-N in the pharmaceutical industrial effluent. The study has wide applications for the treatment of high strength NH_3-N containing pharmaceutical wastewater.

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Figure Legends

Fig.1. Schematic representation of construction of pSC189::miniTn(Gm)

Fig.2. Maximum likelihood phylogenetic tree of *Proteus penneri* with other closely related species on 16S rDNA sequences. Numbers in the nodes are the bootstrap values from 1000 replicates.

Fig.3. Effect of (a) time, (b) pH and (c) temperature and, (d) biomass concentration on NH_3-N removal

Fig.4. FT-IR spectroscopy of (a) untreated sample, (b) effluent treated with wild strain, and (c) effluent treated with T_{55} mutant strain

Table Legends

Table 1: Primers used in this study

Table 2. Kinetic rate constants for the degradation of NH_3-N

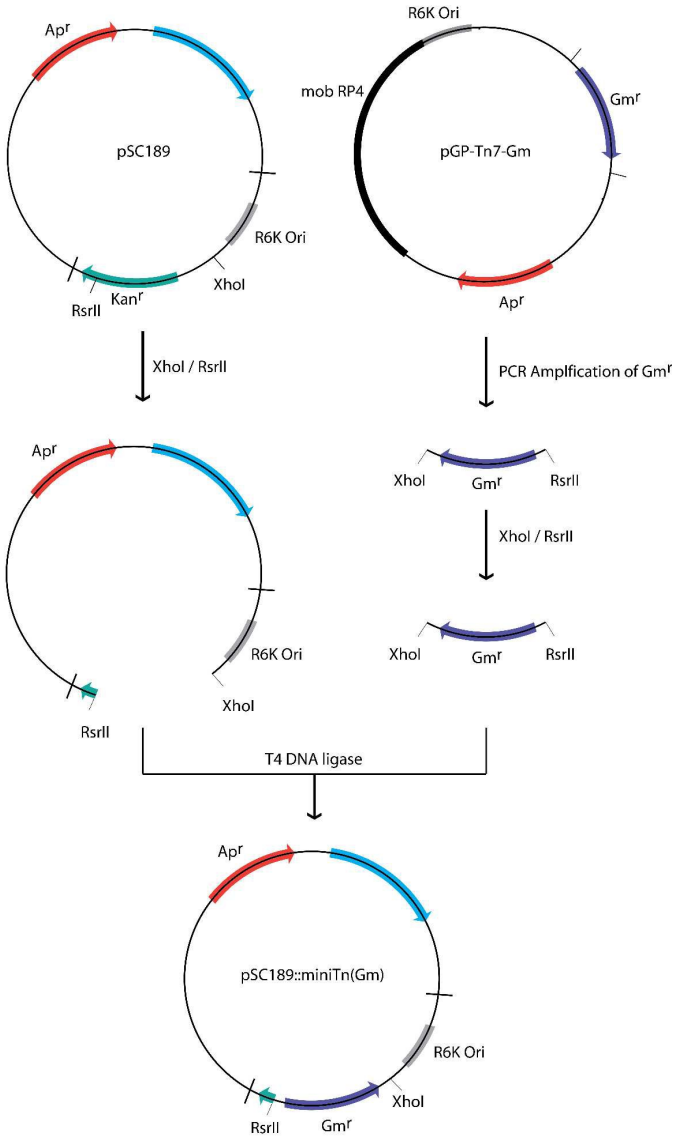

Fig. 1. Schematic representation of construction of pSC189::miniTn(Gm)

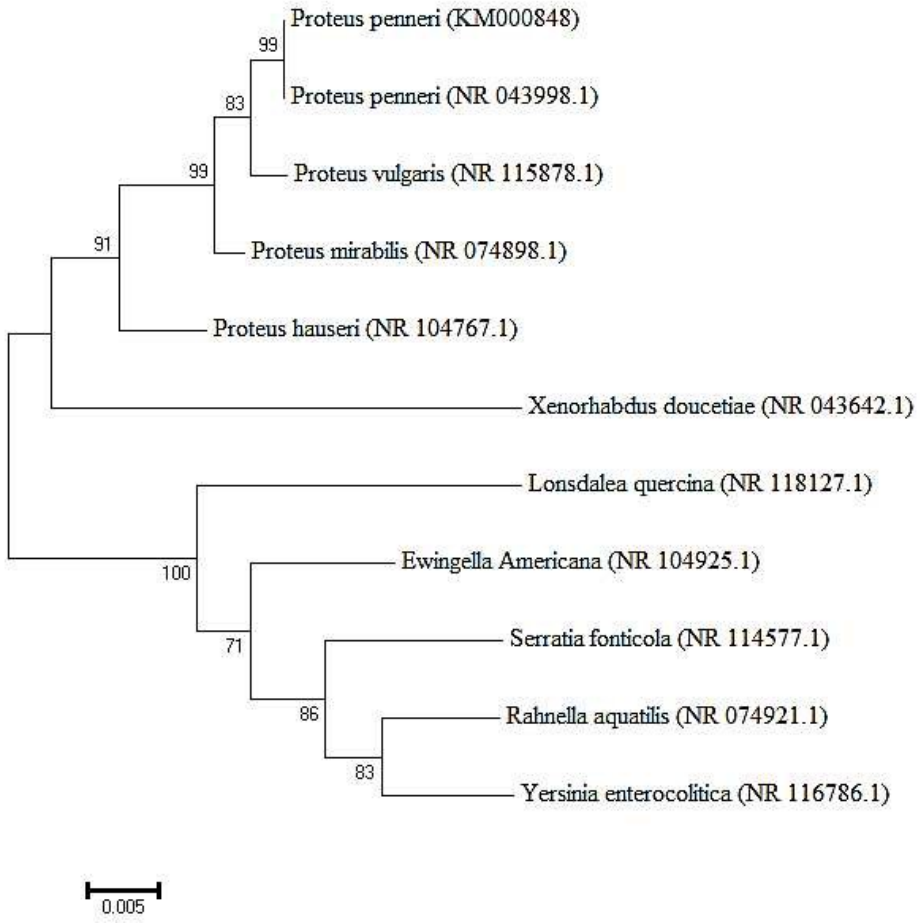

Fig 2. Maximum likelihood phylogenetic tree of *Proteus penneri* with other closely related species on 16S rDNA sequences. Numbers in the nodes are the bootstrap values from 1000 replicates.

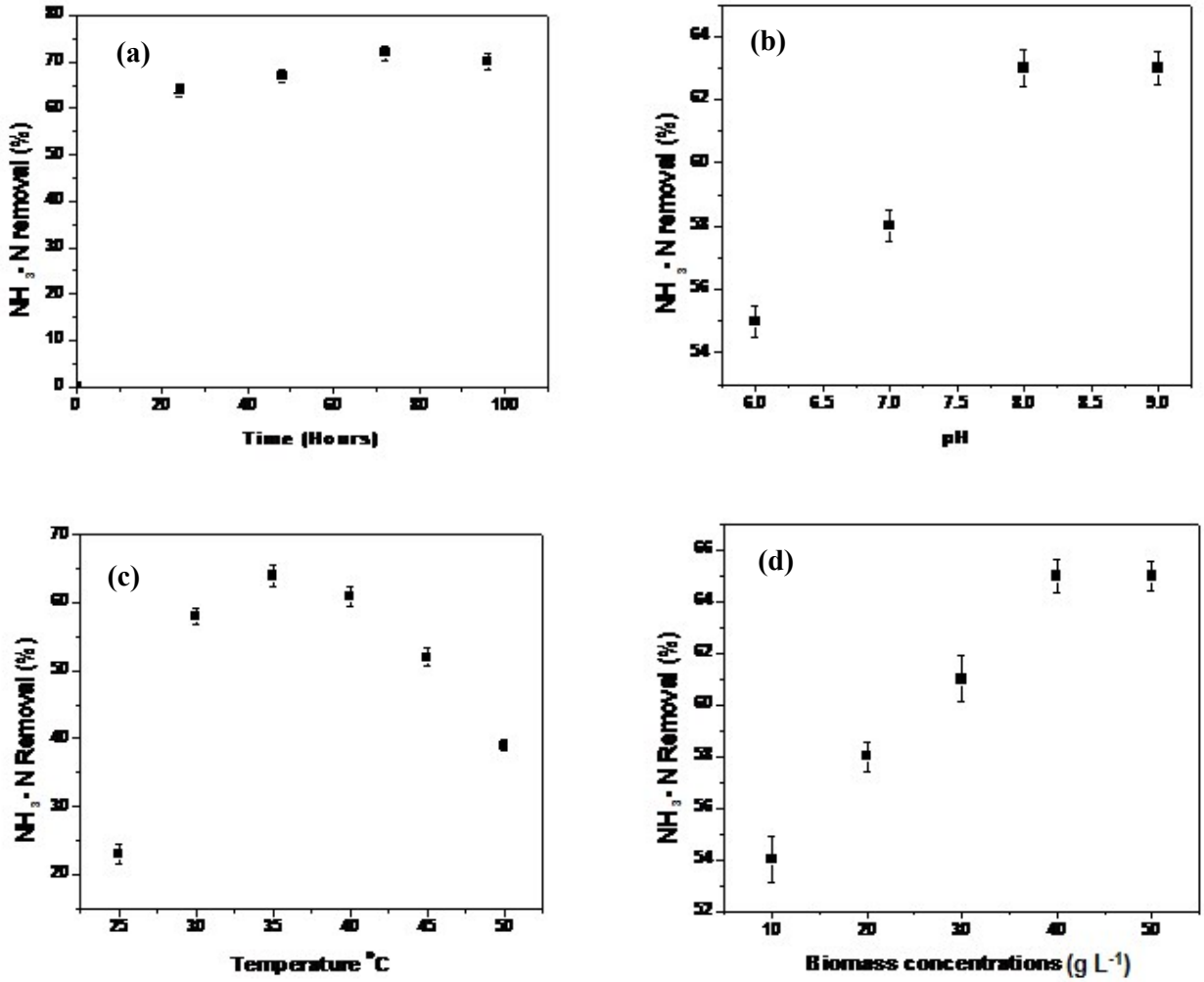

Fig. 3. Effect of (a) time, (b) pH, (c) temperature and, (d) biomass concentration on NH_3-N removal

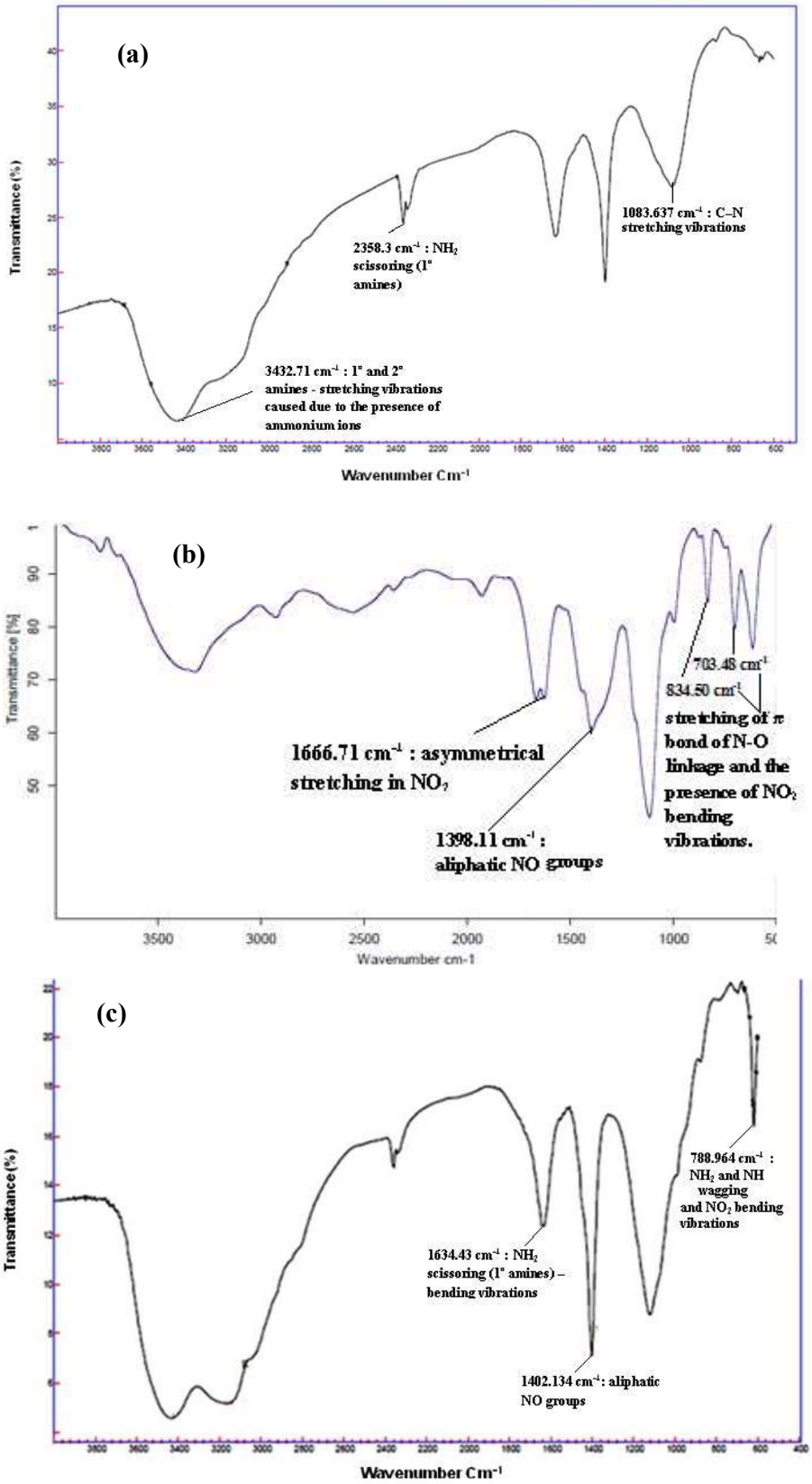

Fig. 4. FT-IR spectroscopy of (a) untreated sample, (b) effluent treated with wild strain, and (c) effluent treated with T_{55} mutant strain

Table 1: Primers used in this study

Name of the primers	Sequence(5′ to 3′)	Function
Gm F	CGGWCCGGAATTGTTAGGTGGCGGTACTTGG	Amplification of Gm^r gene from pGP-Tn7-Gm
Gm R	GAGCTCATGCATGATATCGTCCG CTCGAG	
R6K F	GCCGCTCTAGACCCCTATAGTGAGTC	Amplification of the R6K origin of replication site for the confirmation of transposon integration in the genome
R6K R	GAATTCCTGCACCCTTAATTAACCCCG	

Table 2. Kinetic rate constants for the degradation of NH_3-N

	Pseudo first order		Pseudo second order	
Microbial strain	k_1 (h^{-1})	R^2	k_2 (mg/L/h)	R^2
***P. penneri* (wild type)**	0.028	0.966	2.56×10^{-3}	0.999
Transposan mutant	0.039	0.928	1.75×10^{-3}	0.999

Graphical Abstract