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Biodegradation of Pharmaceutical Waste,4-Nitrophenol by Pseudumonas Putida

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ABSTRACT

The present work was done on " Biodegradation of Pharmaceutical waste,4-Nitrophenol by Pseudumonas putida, in an pharmaceutical laboratory in Chambaghat, Distt.Solan. There is an consistent increase in the use of pharmaceuticals, driven by both drug development and our aging population. It's creating a corresponding increase in the amount of Pharmaceutical waste generated. Pharmaceutical wastes have detrimental effects on the aquatic species and on human health. In pharmaceutical manufacturing plants, the unused or damaged carton boxes, waste aluminium foils, solvents and chemicals used in Quality control department and production department were discarded. Many efforts were done to reduce the production of the waste but it was not possible to completely eliminate these problems. Efficient waste water treatment facilities were necessary for pharmaceutical industry. Here, we analysed the waste water from pharmaceutical, both chemically and biologically. Biological methods were done to identify the bacteria, Pseudomonas putida. Chemical analysis was done with the use of HPLC (High Performance Liquid Chromatography) technique. After performing the analysis, we saw the results. Analysis showed that initially, there was low number of microbes and large concentration of toxic industrial waste in the sample. After 24 hrs we checked the same sample and found that the number of organisms increased and the chemical concentration goes on decreasing. It showed that Pseudomonas putida degraded the chemical 4-Nitrophenol.

KEYWORDS Biodegradation, Pseudumonas, pharmaceutical, Industrial waste, HPLC, 4-Nitrophenol

INTRODUCTION

Pharmaceutical waste is not one single waste stream but many distinct waste streams that reflect the complexity and diversity of the chemicals that comprise pharmaceuticals. Pharmaceutical waste is potentially generated through a wide variety of activities in a health care facility including intravenous(IV) preperations,general compounding,spills/breakage,partially used vials,syringes,unused preperations,unused unit dose repacks,patients personal medications and outdated pharmaceuticals.Interest in the microbial biodegradation of pollutants has intensified in recent years as people started to clean up contaminated environments.These bioremediation and biotransformation methods help to degrade,transform and accumulate a huge range of compounds including hydrocarbons (oil),Polychlorinated Biphenyls(PCB's),Polyaromatic Hydrocarbons (PAH's), pharmaceutical substances and metals.

The elimination of a wide range of pollutants and wastes from the environment is an absolute requirement to promote a sustainable development of our society with low environmental impact.Biological processes play a major role in the removal of contaminants by taking advantage of the catabolic activity done by microorganisms to degrade such compounds. New methodological breakthroughs in sequencing,genomics,proteomics,bioinformatics and imaging are producing vast amounts of information.

Pseudomonas putida is a Gram-negative,rod shaped bacteria having smooth colonies.It is saprophytic soil bacteria. A Pseudomonas strain capable of growing on 4-Nirophenol as a sole source of carbon was isolated from pharmaceutical waste dumpsite after selective enrichment.Experiments revealed that the strain utilizes 4-Nirophenol after 6 hours of adaptation and could degrade upto 2.1 mm of 4-Nirophenol (Asifa Qureshi et al.,2001).

Many pharmaceutical industry processes use halogenated aromatics as starting materials or solvent and these can often exhibit inhibitory effects affecting normal process operation of biological Waste Water Treatment Plants (WWTP's).A mixed culture,initially selected for growth as the sole source of carbon and energy,was enriched with a mixture containing Bromobenzene,Chlorobenzene,Fluorobenzene and Idobenzene. After three weeks of continuous operation in a Stirred tank Bioreactor,none of these halogenated compounds were detected in the growth medium.Mineralization of Bromobenzene,Chlorobenzene and Idobenzene upto their maximum solubility level (in Minimal Salt Media) was achieved while Fluorobenzene was not biodegraded. The complete mineralization of Idobenzene upto 150 mg l-1 (in 18 hours) with complete recovery of iodide is interesting since published literature showed no previous report of similar results. Preliminary characterization has shown that the cultures responsible for Idobenzene and Bromobenzene biodegradations are strains of Pseudomonas putida (Frietas dos Santos,L.M.,1999).

Biodegradation was carried out in bioreactors and the ability to degrade or utilize cellulose was established on the basis of bacterial growth on cellulose medium (filter paper) as sole carbon source and indirectly by the amount of CO2 produced when substrate was seeded with isolates.The microorganisms with cellulose-degrading potential could be cultivated and used as inoculants for field studies like Aspergillus fumigates, Pseudumonas fluorescens, etc.(Bassey,E.B.,2004).

Most antibiotics are metabolized only incompletely by patients after administration and enter the municipal sewage with the patients excretions .Little is known about their biodegradability in aquatic environments and their role with respect to growing bacterial resistance. Therefore,the biodegradability of some clinically important antibiotic drugs as a very first step of an environmental risk assessment was investigated with the Closed Bottle Test (CBT).To access toxicity of the test compounds against aquatic bacteria, a Growth Inhibition Test (GIT) with Pseudomonas putida was conducted. A toxicity control was used in the CBT and the colony forming units (CFU's) were monitored in the test vessels. Only Penicillin G was biodegradable to some degree (27%) even when the test was prolonged from 28-40 days.CFU monitoring revealed high toxicity for sulfamethoxazole whereas ciprofloxacin had a week but significant effect. Therefore, antibiotic drugs emitted into municipal sewage may affect the biological process in Sewage Treatment Plants (STP's).Antibiotic drugs may persist in the aquatic environment and contribute to the increasing resist-

ance of pathogenic bacteria (Al-Ahmad,A.,2004).

In these studies,Solid-phase Extraction,on-line derivatization and measurement by Ion Trap Mass Spectrometry (ITD-MS) were used to investigate the biological degradation of pharmaceutical residues (clofibric acid,ibuprofen, diclofenac).Two metabolites of ibuprofen could be identified on the basis of their mass spectra and comparison with literature data viz. hydroxyibuprofen and carboxuibuprofen (Christian Zwiener.,2000).

The involvement of new technologies and products in the pharmaceutical industry causes some problems with the disposal of highly concentrated wastes .In such a case the anaerobic pretreatment seems to be an advisable technology.The selection of the most suitable equipment to be employed in the anaerobic treatment of a particular substrate strongly depends on the substrate nature and consequently on the limiting steps of the process (Jenicek et al.,1993).

Bioassay techniques for measuring the presence or absence of inhibitory substances are most promising for resolving anaerobic treatment problems because they are relatively simple,inexpensive and do not require knowledge of specific inhibitory substances (Owen et al.,1979).

Also bioassay techniques are essential for determining biodegradability since no chemical procedure is available which distinguishes between biodegradable and non-biodegradable organics (Gledhill,1979).

In 1995,the first International Standard for evaluation of the ultimate anaerobic biodegradability of organic compounds in digested sludge was issued.Present tendency of many researchers is to improve and automate the measurement procedure.

MATERIAL AND METHODS

The efficient waste water treatment facilities are necessary for any pharmaceutical industry. Various methods are used for the treatment of waste water.The choice of method depends upon the nature of the waste,the facilities available,the money available,environmental norms in that region and the feasibility of the treatment method.The following methods were adopted in the treatment of pharmaceutical waste water.

I) PHYSICAL METHODS
II) CHEMICAL METHODS
III) BIOLOGICAL METHODS

I) PHYSICAL METHODS

It includes the following:

Multimedia filteration : This technology reduces BOD in water which is waste,by removing BOD associated with particulate matter.A multimedia filteration system was operated by introducing waste water to the fixed bed of inert granular media. Suspended solid was removed from the waste water by one or more of the following processes like sedimentation and adsorption.

Incineration: Incineration is used in the pharmaceutical manufacturing industry to treat organic and inorganic constituents in waste water.In this,the waste was burnt at very high temperature in the absence of air.This treatment was typically performed in a fixed bed or multiple hearth incinerators equipped with an acid gas scrubber for control of generated hydrochloric acid. Contaminates in the waste water was destroyed by combustion and the remaining water vapour was discharged to the atmosphere.

II) CHEMICAL METHODS

It includes only one method for the treatment of waste water i.e. Use of oxidizing agents like ozone. Ozone gas is highly reactive and was used in the treatment of waste water. Ozone is effective in oxidizing antibiotics,drugs etc. Also, the combined treatment of hydrogen peroxide and UV radiations reduces the amount of diclofenac present in waste water.

III) BIOLOGICAL METHODS (BIOREMEDIATION)

Biological methods involved the Use of microbes for the treatment of waste water.Microbes convert the waste into some other form.Biodegradation depends upon a number of factors like stereochemistry of the compound,toxicity of the compound and presence of other compounds and their concentrations.It also depends on the efficiency of the microbial strains used during the degradation like pH,temperature,retention time etc.

The waste water sample 100ml was collected from the drain of the pharmaceutical unit in the sterilized 250mi conical flask.The flask was opened only near the sample collection area and immediately it was closed.Then it was stored in incubator for analysis.The analysis methods were as follows:

Biological Analysis
Chemical Analysis
Biological Analysis

Biological processes play a major role in the removal of contaminants and they take advantage of microbial activity to degrade such compounds.Biological Analysis was done in several steps.These are discussed one by one below:

Isolation: Isolation was done by Pour Plate Method (PPM) using Nutrient Agar plates.Sterilization of four petriplates was done.After sterilization,1ml water from the collected water sample was taken with the help of 1ml micropipette and put into three petriplates.One plate was kept control.Then the plates were kept in incubator at 370C for 12 hours.

Identification: After 12 hours of incubation,the petriplates showed the bacterial growth.We only took mostly present bacterial colonies for further analysis.These colonies were only analyzed for identification by biochemical tests.Five different kinds of selective media were used for culturing selective microorganisms.The media used were Brilliant Green Agar for Salmonella species, Cetrimide Agar for Pseudomonas species, Fluid Lactose Medium for coliform group, Potato Dextrose Agar for Yeasts and Moulds and Soyabean Casien Digest Agar for fastidious and non-fastidious microorganisms.

Now, the mostly present colonies were streaked on above mentioned medias.After streaking,the plates were incubated for 12 hours for growth.After incubation,only Cetrimide Agar plate showed the growth of colonies,which indicated that Pseudomonas species might be present.Then the colonies were biochemically identified.After biochemical analysis,the unknown colonies were identified as Pseudomonas putida.

Pseudomonas putida shows following biochemical tests:

Biochemical Tests ResultsFructosePositiveGlucosePositiveGalactoseNegativeMannitolNegativeMaltosed (variable)MalateNegativeXylosed (variable)PhenolPositiveHydrolysis of GelatinNegativeHydrolysis of StarchNegative DenitrificationNegativeFatty acid ContentTracesMolecular % G+C Content60.7% - 62.5%
Initial counts (IC) by Plate Count Method (PCM): After identification using Plate Count Method (PCM), the Initial counts of bacterial colonies could be found.In this method, 1ml water from the collected water sample was taken with the help of 1ml micropipette and put into three petriplates.Added 20ml of sterile Cetrimide Agar in three petriplates and One plate was kept control.Allow the plates for solidification.Then the plates were kept in incubator at 370C for 12 hours.After incubation, Initial count will be calculated and noted the values.

Further Analysis: The same procedure as above in the IC was done in 24 hours interval,48 hours interval,72 hours interval,96 hours interval and 120 hours interval.Every hour's interval ,the CFU was calculated and observations were noted.The same waste water samples were taken in every time intervals.

Final counts (FC) by Plate Count Method (PCM): After 5 days of incubation,the same waste water was analysed by PCM

and CFU was also calculated.This will be final counts of the Pseudomonas putida present in the waste water. Pseudomonas putida used 4-Nirophenol as a sole carbon source by the degradation process.

2.Chemical Analysis

Chemical Analysis revealed that the waste water from the pharmaceutical industry have little concentration of Nitrogenous compounds,Nitroaromatic compounds,Fluorinated compounds,amines and Phenolic compounds.These chemical compounds were toxic to the environment as they affect the microorganisms as well as environment.These compounds also reduces the soil fertility and makes ground water polluted.So reducing these chemical compounds was an important thing. Chemical Analysis was done in several steps.These are discussed one by one below:

Analysis of waste water: The same waste water sample previously collected for Biological method was used for Chemical Analysis.

Identification of chemical compounds by using HPLC: The experiments were performed usingspecific methods for comparison studies. The waste water sample collected was identified for chemicals by High Performance Liquid Chromatography (HPLC-Analytical Instruments Solutions,Model AIS 2600 LC). Seperations were performed on a C-18 Column (make Grace USA,250X4.6mm).The mobile phase was used as Methanol (550ml) and Water (450ml).The flow rate was 1ml/min. and the temperature was ambient at the wavelength of 234nm. Firstly,using a standard of 4-Nirophenol compounds,standard peak for 4-Nirophenol was plotted.This standard will be used for the further analysis.The unidentified peaks were also formed.

Initial concentration of 4-Nirophenol: After the standard peak,1ml waste water was taken and diluted with mobile phase.Then injected 20µml of it into HPLC.Then the peak of 4-Nirophenol was formed.The area shown in the peak was calculated and concentration of 4-Nirophenol was noted.The concentration of 4-Nirophenol in initial was more but later it decreased due to the Pseudomonas putida degradation.

Further Analysis: The same procedure which was done in the Initial Concentration was done in 24 hours interval,48 hours interval,72 hours interval,96 hours interval and 120 hours interval. Every hour's interval, the concentration of 4-Nirophenol was calculated and observations were noted.The same waste water samples were taken in every time intervals. Gradually, the concentration of 4-Nirophenol will be reduced due to the Pseudomonas putida degradation.

Final concentration of 4-Nirophenol: After 5 days, the final concentration of 4-Nirophenol will be calculated.The concentration amount will be reduced due to degradation of this compound by Pseudomonas putida .The concentration of 4-Nirophenol will be more decreased.The amount of the final concentration was very negligible.This will not pollute the environment.

Thus the biodegradation took place in the waste water of the pharmaceutical industry.Presence of the chemical compounds in this waste water will be degraded by the microorganisms. So,the waste water will not pollute the environment.

RESULTS AND DISCUSSION

RESULTS

After the completion of all the analysis,we got the results according to the degradation of 4-Nitrophenol by Pseudumonas putida .The bacterial growth was increased due to the degradation of 4-Nitrophenol.The growth of Pseudumonas putida was very high which showed it utilize the 4-Nitrophenol as sole carbon energy.The analysis was done by the equal interval of timings like 24 hours to 120 hours means fifth day from the starting day. Initial counting of Pseudumonas putida showed a less amounts i.e 65 CFU/ml of waste water sample.It proved that the pharmaceutical waste water had very less amount of microorganisms.On the other hand,Chemical analysis proved that the 4-Nitrophenol concentrations were 99.56%.This amount was somewhat much in the waste water which come to the drain of pharmaceutical industry from quality control laboratory.

After 24 hours,analysis of waste water showed the Pseudumonas putida growth of 137 CFU/ml and the concentration of 4-Nitrophenol was 69.90%.The bacterial growth slightly increased here.After 48 hours of incubation,the bacterial growth was increased to 248 CFU/ml and the concentration of 4-Nitrophenol was 45.34%.The concentration of the chemical compound will be decreased due to the utilization of Pseudumonas putida .Then after 72 hours of incubation,once again the waste water was analyzed and the concentration of the compound and colonies of Pseudumonas putida was observed which was like 307 CFU/ml colonies and 21.72% of 4-Nitrophenol concentration.After 96 hours incubation,the observations was 392 CFU/ml colonies and 16.14% of 4-Nitrophenol concentration.Finally,after 5th day, Pseudumonas putida showed the growth of 431 CFU/ml and 3.62% concentration of 4-Nitrophenol.The 4-Nitrophenol will be much reduced from the initial amount to the final concentration.This proved that the Pseudumonas putida uses the 4-Nitrophenol and degraded it.

The waste water from the pharmaceutical will be treated by the Pseudumonas putida and just like other microorganisms. The microbes can eat waste and pollution.The waste water from the industry will degrade by the microflora present in the environment.

Following table showed the degradation of 4-Nitrophenol by Pseudumonas putida

Pseudumonas putida growth and 4-Nitrophenol degradation-Time intervals in hoursConcentration of 4-NitrophenolPseudumonas putida growth%CFU/mlInitial raw water99.5665After 24 hrs69.90137After 48 hrs45.34248After 72 hrs21.72307After 96 hrs16.14392After 120 hrs3.62431

DISCUSSION

Biotreatment, the processing of waste using living organisms,is an environmentally friendly relatively simple and cost effective option.Pharmaceutical waste management needs a unique procedure.The main principles of these studies was :

Degradation of Pharma Waste by biological methods Management of regulated hazardous pharmaceutical waste Minimization of pharmaceutical waste The involvement of new technologies and products in the pharmaceutical industry causes some problems with the disposal of highly concentrated wastes.

Bioremediation refers to the conversion of toxic environment containing compounds into harmless environment by way of microbial digestion.In most situations,the microorganisms naturally present in the soil and groundwater are capable of degrading the contaminating compounds.The bioremediator must enhance the growth of naturally occurring microorganisms.The materials employed in the present invention deliver nitrogen,phosphorus and sulfur as macronutrients to the bioremediation microorganisms.

Microorganisms are essential for our existence.They are ubiquitous.They are responsible for recycling of nutrients in soil & purifying water.They detoxify acid mine drainage and other toxins that we dump into the soil and water.Here,the study about the pharmaceutical waste water analysis had been done by both chemical and biological methods.It had been reported that the isolated microorganism degraded high concentration of 4-Nitrophenol as a sole source of carbon.The microorganism isolated by both the methods was Pseudumonas putida .It can degrade 4-Nitrophenol within 5 days.The whole analysis

showed that the degradation of 4-Nitrophenol depend upon the biomass of the bacterial counts.

The whole study revealed that the Pseudumonas putida involved the degradation of 4-Nitrophenol.The microorganism will degrade the chemical compounds and it clears the environments.

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