



Isolation and Characterization of Protease producing Fungi for X-Ray plate Bioremediation

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Abstract: - Every year the manufacturing of X-ray film sheets worldwide can use up to 1000 tons of total silver wasted using these chemically manufactured films. To avoid waste and promote re-use of the plates, biological methods are used to study the effect of disposed X-rays on the production of two types of enzymes using the power of microorganisms. The biological agents like a series of fungi may be used so that isolation of the protease and usage of it may be shown. The dumping ground was located with an area that had pretty huge reserves of X-ray films and other medical items. Proper care was taken in handling and separation of the soil from the regions that were demarcated from the five different sites of the NDDM ground. The isolates were collected and checked for protease activity and then isolates that showed best results were marked as T1 and T2. First, the production of the protease enzyme following the use of disposed X-rays as a carbon source. Secondly, nitrate reductase enzyme may help in the reduction of the silver nitrates that are present in the X-Ray films. All discarded X-rays are brought into the substrate. Different fungal isolates were collected from the region around the North Dum Dum Municipality Dumping Ground. The different amounts of the isolates were taken and they were utilized in the making of the experimental models that used to verify the hypothesis and untapped or unconventional methods of using the fungi as potential sources cleaning x-ray films naturally. The isolated enzyme was then used for further series of experiments, and the hypothesis regarding the usage of the fungi was used and verified, the experiments were conducted in duplicate sets for confirmation.

Keywords – Bioremediation, X ray, Nitrate reductase, Fungi, Protease.

Introduction: -

The most important global concern for present day world is pollution. Due to increased industrialization and urbanisation, for the fulfilment of human demand the rate of pollution has increased and thus resulting in adverse effect to the human health and welfare. Due to increase in demand of resources, severe environmental problems has arouse like the release of different types of waste material to the soil ,water, air (Fontenelle et al., 2019; Jiang et al. 2008). However radioactive wastes like used x-ray plates, nuclear waste material discharged from the industries etc has been a serious environmental problem (Vandana et al.). In order to deal with land contaminated with radioactive wastes, several researches are being conducted which are environmentally safe and publicly acceptable methods.

However the most compatible method for disposal of these radioactive wastes include bioremediation where the word 'bio' means living being and 'remediation' means solution or to solve the problem. Bioremediation is the process of using living microorganisms to degrade environmental pollutants into less toxic form or nontoxic form. Bioremediation, on the basis of area of waste treatment are of two types-

1. Insitu Bioremediation
2. Exsitu Bioremediation.

Insitu Bioremediation method is cost effective and is the most common type used for waste disposal at the pollutant site (Shashikumar et al., 2003).

Bioremediation is carried out by microorganisms (mainly bacteria and fungi) , where these organisms act against the pollutant through their ability to breakdown waste material enzymatically. Fungi are the best choice for bioremediation because of its major role as decomposer. Fungi have the ability to degrade waste material through extracellular and intracellular enzymes (Deshmukh et al., 2016). Fungi is frequently used in various laboratories and industries because e enzymes produced by fungi often has an advantage of easy mycelium separation by simple filtration (Devi et al., 2008). Also fungi can be easily grown in an inexpensive media.

Both fungi and bacteria produce certain enzymes that promote the degradation of the waste pollutants. The enzymes include oxygenase, peroxidase, laccase, cellulase, lipase, protease etc. Protease hydrolysis the breakdown of proteinaceous substances by breaking down the peptide bonds (Chandrakant et al., 2011).

Protease is one of the largest industrially and commercially important enzymes. It accounts for 60% of the worldwide sale of enzyme (Nurullae. A et al., 2011). Protease can be of two types- endopeptidase and exopeptidase. There are several applications of protease enzyme which involves usage as detergent additives. It improves cleaning and removes dyes from cloth and also function in serving the extraction of gelatin from used x-ray plates.

It is found that after use of x -ray plates, it has been disposed off and this may cause pollution and have adverse effect on human health. In this present study an effort is done to isolate and characterise some microorganisms in the form of fungi from NDDM Dumping Ground which has the ability to produce protease enzyme such that it can solubilize the gelatin substance from the x -ray plate and thus can be used for further bioremediation studies.

2. Procedure: -

2.1 Isolation of protease producing strains

The protease producing strains were isolated from the area of NDDM Dumping ground and then they were collected as soil samples from 5 different areas across the location that showed maximum amounts of medical X-ray and other utilities being thrown away. The soil samples were collected in a sterile plastic bag from those 5 different sites and then they were all mixed up into one conjugated sample (Yusha'u *et al.* 2021, p.6).

The soil samples were taken and then serially diluted up to 5x dilutions prepared with sterile water, and each of the dilutions were added to tubes and then incubated in a fresh potato dextrose agar. The samples were all processed according to the highest degree of sterility and then allowed to grow for 48 hours (Shah *et al.* 2019, p.5). The growth of colonies of various fungi were noticed and the colonies were marked according to their quality of growth and distribution, in total there were 5 colonies that were marked to be used in further tests.

2.2 Microscopic examination of the fungal strains isolated

The isolated samples were taken and then microscopic examination was performed for each of the samples by the virtue of Lacto-phenol cotton blue staining method (Zieliński *et al.* 2019, p.09449). This method of staining involves the usage of lacto-phenol and cotton blue after picking the samples they were properly placed in a clean grease free slide and teased with the above mentioned stains (Poojary *et al.* 2019, p.277). The samples were observed under the 450x magnification under microscope (Olympus MLX-B).

2.3 Solubility indices of the isolated fungi were assessed to get best colonies

To isolate the best colony among the colonies that have been obtained, all the colonies were assessed according to their capability to degrade the milk proteins and this was done using a casein agar method. Milk Casein Agar was made and all the 5 colonies were incubated in the centre of the plates by taking a massive chunk of already grown fungi's from a separate plate (Madelin *et al.* 2020, p.383). The clearance was measured such that more even clearance is associated to

higher activity of the protease produced by the isolates. The samples that have shown superior zone of clearance were taken for further tests and rest of the samples were discarded.

2.4 Calculation of the zone of clearance

The zone of clearance were all measured and then using the formula for zone of clearance,

Zone of clearance = (COLONY +ZONE diameter)/ COLONY diameter.

The measured zones indicated that some of the colonies were better suited for the clearance and other samples were withheld in the experiment.

2.5 Isolation of fungal genomic DNA and identifying the samples

Two samples were found to be best and they were taken in this step. The samples were first grown in larger volumes and then the isolation was conducted by the usage of methods involving, mycelia DNA extraction.

1. Colonies of the fungus were taken using sterile mounting and isolation techniques and then they were cut into smaller pieces. Mycelium was mainly taken for this purpose to obtain maximum amount of DNA from the samples. The mycelia were then again incubated according to the filtration and inoculation techniques and the resulting mycelium chunks were harvested (Snyman *et al.* 2019, p.5525).
2. The mycelium volumes from two samples were separately freeze dried and then the mycelium bits were added to a buffer with lysine tris-Acetate buffer (40mMol/l tris acetate, 20 mMol/l Sodium acetate, 1mMol of EDTA, 1% w/v SDS and the resultant pH was 7.8). The freeze dried samples were then inserted and shaken in a rotary shaker so that a vehement display of frothing was noticed (Zanutto-Elgui *et al.* 2019, p.830).
3. The step was repeated for more than 15 times and each time the mycelium walls were penetrated by the mixture. The protein and other cellular debris were precipitated out using the 5m/l of NaCl solution (Badet *et al.* 2020, p.15). The resultant suspension was again ultra-centrifuged so that all the debris and proteins were settled easily and overall supernatant generated was removed from the mixture and then phase extraction and phenol based extraction of DNA was done according to the conventional methods (Chimbekujwo *et al.* 2020, p.00398).
4. The isolated DNA was then purified and it was used in PCR for amplification of the DNA matter. The reverse and forward primers were also designed by the similar methods and overall 30 cycling were conducted in a conventional PCR.
5. ITS regions were amplified by the PCR a discrete band were tried to be maintained.
6. Forward and reverse DNA sequencing was carried out using ITS 1 and ITS 4 primers respectively. PCR machine that was used in this case is a *BDT v3.1 Cycle sequencing Kit on ABI 3730xl Genetic analyzer*.
7. Consensus regions were generated and data aligner software was used to align the data properly.
8. The ITS regions were also used to carry out BLAST with NCBI GenBank database to be the information retrieval site.
9. Multiple alignments were performed and Clustal omega distance matrix was also generated.
10. The phylo-genetic tree analysis was conducted using the MEGA 7 software (Zanutto-Elgui *et al.* 2019, p.830).

2.6 Clearance and bioremediation of the used X-Ray plates

The bioremediation of the X-ray plates were chosen as they are wasted in large amounts and hence we require a few methods to conserve those (Kumar *et al.* 2019, p.124567). The bioremediation is done by taking a 4cm by 4cm strip of X-ray plate then, it was sterilized using 95% ethanol and maintaining sterility it was immersed in a covered plate that contained PD broth at physiological pH and along with that fungi no. T1 and T5 were also utilized. T1 and T5 showed best results during protease screenings among the 5 isolates (Paria and Chakraborty. 2019, p.7). The plates were then immersed and the incubation time of 1 week was allowed. The plates were then removed from the broth and cleaned with distilled water and the clearances were noted such that all black X-ray film coatings were absent. The one showing best activity regarded as the final sample for carrying out other future procedures (Silva *et al.* 2019, p.1555).

3. Results: -

3.1 Observation of protease activity in Milk Agar plate

Initially we have isolated 5 different colonies depending on colonial dissimilarity in Potato dextrose agar media and later all of them were tested for protease production in Casein Milk agar plates. After growth for 48 hours in Milk agar plates a clear zone of hydrolysis was observed by both TI and TII isolates. This confirmed that our isolated fungi has the extracellular protease activity and hence can be used for further studies.



Fig1:- Zone of clearance in milk agar plate by TI



Fig2:- Zone of clearance in milk agar plate by TII.

3.2 Calculation and Determination of Solubility index

Solubility Index was calculated from both TI and TII using the formula.

SAMPLE	SOLUBILITY INDEX
TI	3.05
TII	1.10

As it has been observed that the solubilization index was higher for TI sample compared to TII sample, further study were conducted by using TI sample.

3.3 Morphological identification by lactophenol cotton blue staining:-

After isolation of distinct colonies, microscopic observation was done by taking a small amount of culture from the petriplate and stained it by Lactophenol cottonblue on the slide. The slide is then observed under light microscope for distinct morphological features at 450X magnification.

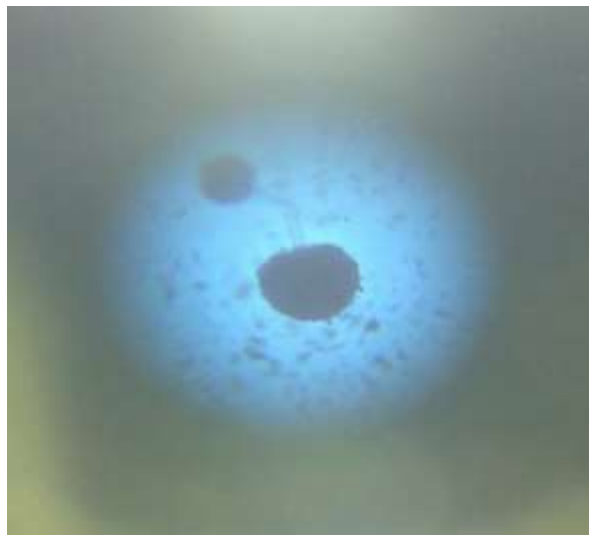


Fig 3. The morphological features are under light microscope(450X).The morphological feature it showed was similar to that of *Aspergillus sp.*

3.4 Identification of Genomic DNA

1. Fungal genome was isolated and named as BAA1. The sample was run on agarose gel, a single molecular weight has been observed.
2. Fragments of Internal Transcribed Spacer region was amplified by PCR and a single discrete PCR amplicon band of ~700 base pair was observed when resolved on Agarose gel.

gDNA and ITS Amplicon QC data:

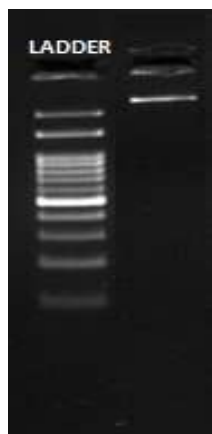


Fig:4-gDNA

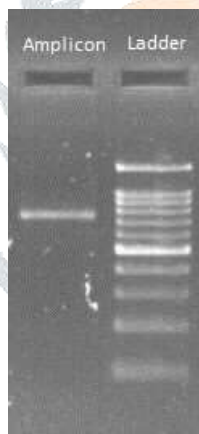


Fig:5-PCR amplicon

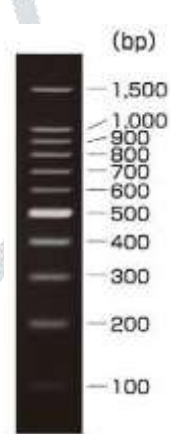


Fig:6-Marker DNA

Forward and reverse DNA sequencing reaction of PCR amplicon was carried out using Sanger sequencing. Using BLAST consensus sequence of the PCR amplicon was generated.

3.5 Construction of Phylogenetic Tree

Multiple alignments were further carried out with the maximum identity score using ClustalW and phylogenetic tree is generated using Mega7 software.

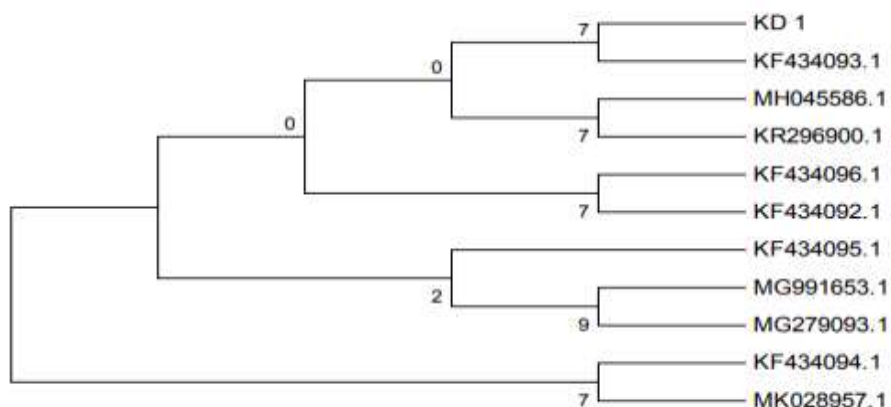
Phylogenetic Tree:

Figure. Molecular Phylogenetic analysis by Maximum Likelihood method

Interpretation- Sample KD1 showed higher similarity with *Aspergillus tubingenensis* based on nucleotide homology and phylogenetic analysis.

4. Application: - Reuse of waste X-ray plate

The fungi has been inoculated in a suitable Czapekdox broth and after incubation for 48 hours the sample has been taken and centrifuged at 6000rpm for 10 minutes. The cells are separated from the supernatant. The supernatant is then passed through 0.22µm membrane filter so that it can trap cell and allow the cellular content to pass through the membrane. The filtrate obtained is then tested against the used x-ray film. It showed protease activity by removal of gelatinous coating from x-ray film.



Fig 7. Control (Untreated) X-Ray plates



Fig 8. Effect of KD1 crude enzyme

Fig 8. clearly indicates the removal of gelatin from X ray plates thereby indicating protease activity of the fungal isolate.

5. Discussion: -

Protease are the enzymes that are found widespread in nature and microbes are considered to be the preferred source of these enzymes due to their rapid growth. The only disadvantage these microbes has it the production of enzyme at a very low concentration. The improvement of this problem can be done by growing these protease producing

microorganisms in the laboratories and industries under optimal conditions. Any advantage of growing these microorganisms in the laboratories is that they can be genetically modified to produce modified enzymes which have more efficacy and potentials to carry out certain enzymatic reactions. However fungi is much more preferred choice for protease production due to their ability to grow in an inexpensive media under varying environmental conditions (Haq et al., 2006). In Chinmasamy et al., 2011 it was documented that *Aspergillus flavus* strain showed maximum protease production at pH 4 and temperature 30 degree Celsius whereas in Amarnath et al., 2012, it was found that the strain can produce Maximum protease at 27 degree Celsius temperature and pH 6.

The usage of protease has increased as detergent additives and several other applications which includes bioremediation of radioactive wastes like used x-ray plates and removal of gelatinous strip from the plate. These radioactive wastes need to be decomposed so that they produce less harm to living welfares (Chung *et al.* 2019, p.721).

6. Future prospects: -

The future prospect for this study revolves around the isolation of genes that helps in the solubilizing of X—ray films and other potential sources that may harm the environment. The effective way to introduce such capacity to the strain T2 will be to identify the particular gene that is responsible for the production of the protease (Kjærboelling *et al.* 2020, p.10). The gene may be multiplied or cloned into some vectors and then over-expressed so that there is a high amount of clearances and bioremediation in this regard. The genetic study on the usage of the perfect combination of genes that will be responsible for the removal of other wastes may also be utilized in this perspective (Anupma *et al.* 2020, p.905). Overall a favorable amount of resources may be utilized in developing this strain into a better and high potential waste remover. The usage of eukaryotic means is even more helpful in this regard as they are not that pathogenic and the production ratio may be balanced so that our desired output may be increased (Riach *et al.* 2020, p.229). One may assume that a biological component in the processing of wastes is much better than chemical means that makes much worse outcomes and is highly expensive.

References:-

1. Anupma, A. and Tamang, J.P., 2020. Diversity of filamentous fungi isolated from some amylase and alcohol-producing starters of India. *Frontiers in microbiology*, 11, p.905.
2. Badet, T., Oggenfuss, U., Abraham, L., McDonald, B.A. and Croll, D., 2020. A 19-isolate reference-quality global pangenome for the fungal wheat pathogen *Zymoseptoria tritici*. *BMC biology*, 18(1), pp.1-18.
3. Chimbekujwo, K.I., Ja'afaru, M.I. and Adeyemo, O.M., 2020. Purification, characterization and optimization conditions of protease produced by *Aspergillus brasiliensis* strain BCW2. *Scientific African*, 8, p.e00398.
4. Chung, D., Kim, H. and Choi, H.S., 2019. Fungi in salters. *Journal of Microbiology*, 57(9), pp.717-724.
5. Deshmukh, R., Khardenavis, A. A., & Purohit, H. J. (2016). Diverse metabolic capacities of fungi for bioremediation. *Indian journal of microbiology*, 56(3), 247-264.
6. Devi, M. K., Banu, A. R., Gnanaprabhal, G. R., Pradeep, B. V., & Palaniswamy, M. (2008). Purification, characterization of alkaline protease enzyme from native isolate *Aspergillus niger* and its compatibility with commercial detergents. *Indian journal of science and technology*, 1(7), 1-6.
7. Fontenelle, F. R., Taniguchi, S., da Silva, J., & Lourenço, R. A. (2019). Environmental quality survey of an industrialized estuary and an Atlantic Forest Biosphere Reserve through a comparative appraisal of organic pollutants. *Environmental Pollution*, 248, 339-348.
8. Jiang L, Young MH, Hardee K (2008) Population, urbanization and the environment. *World Watch*
- Karigar, C. S., & Rao, S. S. (2011). Role of microbial enzymes in the bioremediation of pollutants: a review. *Enzyme research*, 2011.
9. Kjærboelling, I., Vesth, T., Frisvad, J.C., Nybo, J.L., Theobald, S., Kildgaard, S., Petersen, T.I., Kuo, A., Sato, A., Lyhne, E.K. and Kogle, M.E., 2020. A comparative genomics study of 23 *Aspergillus* species from section *Flavi*. *Nature communications*, 11(1), pp.1-12.

10. Kumar, V. and Dwivedi, S.K., 2019. Hexavalent chromium reduction ability and bioremediation potential of *Aspergillus flavus* CR500 isolated from electroplating wastewater. *Chemosphere*, 237, p.124567.
11. Madelin, T.M. and Madelin, M.F., 2020. Biological analysis of fungi and associated molds. In *Bioaerosols handbook* (pp. 361-386). CRC Press.
12. Mukhtar, H., Ali, Z., & Riaz, N. (2004). Protease biosynthesis by mutant strain of *Penicillium griseoroseum* and cheese formation. *Pakistan Journal of Biological Sciences (Pakistan)*.
13. Paria, K. and Chakraborty, S.K., 2019. Eco-potential of *Aspergillus penicillioides* (F12): bioremediation and antibacterial activity. *SN Applied Sciences*, 1(11), pp.1-8.
14. Poojary, S., Miskeen, A., Bagadia, J., Jaiswal, S. and Uppuluri, P., 2019. A study of In vitro antifungal susceptibility patterns of dermatophytic fungi at a tertiary care center in Western India. *Indian journal of dermatology*, 64(4), p.277.
15. Riach, M.B. and Kinghorn, J.R., 2020. Genetic transformation and vector developments in filamentous fungi. In *Fungal Genetics* (pp. 209-233). CRC Press.
16. Roh, C., Kang, C., & Lloyd, J. R. (2015). Microbial bioremediation processes for radioactive waste. *Korean Journal of Chemical Engineering*, 32(9), 1720-1726.
17. SA, A., SINGH, A., GARG, S., KUMAR, A., & KUMAR, H. (2012). Screening, isolation and characterisation of protease producing moderately halophilic microorganisms. *Asian Jr. of Microbiol. Biotech. Env. Sc.*, 14(4), 603-612.
18. Sasikumar, C. S., & Papinazath, T. (2003, December). Environmental management: Bioremediation of polluted environment. In *Proceedings of the Third International Conference on Environment and Health* (pp. 465-469). Department of Geography, University of Madras, Chennai and Faculty of Environmental Studies, York University, New York.
19. Shah, S., Shrestha, R., Maharjan, S., Selosse, M.A. and Pant, B., 2019. Isolation and characterization of plant growth-promoting endophytic fungi from the roots of *Dendrobium moniliforme*. *Plants*, 8(1), p.5.

