RESEARCH ARTICLE

Soil fungi, *Aspergillus niger* NAAC, as environmental pollution clean-up agent against Progesterone: Remediation strategy and preparation of Bioformulation

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Abstract: The presence of hormones, drugs and chemicals in the environment are disrupting the ecosystem. The existence of chemicals in the environment is a threat to the ecosystem as they have dangerous effects on animals, plants and microbes. Progesterones are steroid hormones used for human contraceptives and therapeutic purposes as well as promoting animal growth. The cases of consumption of progesterone for medical purposes are much higher than estrogens, however, much studies related to estrogens have been conducted thereby neglecting the effects of progesterone. This invention focuses on removal of progesterone using fungal strain. *Aspergillus niger* NAAC efficiently degraded the progesterone content and transformed it into non-toxic end product. The uniqueness of this study involves preparation of a formulated product which would store the fungal strain and maintain its viability. The bioformulation was prepared using used vegetable cooking oil mixed with water as carrier. The bioformulation would reduce the efforts required to isolate the microorganism for regular usage and can be commercialized for large scale applications. To determine the storage conditions of bioformulation, various parameters were analysed which showed that storing the formulation in air tight container at room temperature would result in maximum longevity of the efficient strain.
Keywords: Fungal strain, hormones, steroid, vegetable cooking oil

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1 Introduction

Endocrine disrupting contaminants are recent threats in causing an imbalance in the ecosystem. The natural progesterone regulates the functioning of female reproductive system. The steroid progestational ketone, progesterone, in used extensively in hormone replacement therapy. It enters the environment through human excretion and further contaminates the surface water. Application of microbial techniques in biodegradation of steroids are mostly focussed based on estrogen. However, recent days, studies are conducted being conducted focussing on elimination of progesterone by microbial cells. Bacterial strains, such as, *Bacillus* sp. and *Comamonas testosteroni* have been reported earlier for the breakdown of progesterone under aerobic conditions (Misra et al., 2021). In another study, effect of *Rhodococcus* sp. was analysed on progesterone degradation which concluded that microbial degradation of pollutants effectively results in production of eco-friendly non-toxic end products (Yu et al., 2018). Though not much studies have been conducted addressing progesterone degradation using fungi, one previous report claimed *Aspergillus niger* strain VKPM F-1069 was able to tolerate the progesterone concentration in medium and successfully declined its concentration (Savinova et al., 2019). In recent days, *Talaromyces* sp. and *Penicillium citrinum* H7 were also reported to be able to degrade progesterone (Dos Santos et al., 2022). However, even though these microbes were claimed for effective biodegradation, no studies included the management strategy of these effective microbial strain. To avoid prolonged process of isolation, is this study, we have focussed on preparing a bioformulation of the effective microbial strain which can be used directly for remediation technique.

2 Materials and Methodology

2.1 Isolation and identification of progesterone degrading fungus

The isolation of the progesterone (PGT) degrading fungal isolate from the sewage soil sample was carried out using enrichment technique. The sewage soil sample was incubated

in the enrichment medium supplemented with PGT. Screening of the potent fungal isolate from the enrichment culture was accomplished by gradient dilution plate method with 100 mg/L potato dextrose agar (PDA^R) medium. Fully grown fungal colonies were further screened and preserved to maintain pure culture. The mycelial growth of the isolated fungal strains was compared by inoculating in potato dextrose broth (PDB), Czapek Dox Broth (CDB), Sabouraud's Dextrose Broth (SDB), and minimal (M1) medium to estimate the suitable growth medium.

The morphological characteristics of progesterone degrading fungus was observed under light microscope using lactophenol cotton blue stain. The taxonomic identification of the fungal isolate was determined by 18S rRNA gene sequencing. Fungal chromosomal DNA was extracted with the help of spin column kit. Amplification of fungal 18S rRNA gene (1500 bp) was carried out using polymerase chain reaction in a thermal cycler followed by purification using Exonuclease I - Shrimp Alkaline Phosphatase (Exo-SAP) (Darby et al., 2013). Sequencing of the purified amplicons by Sanger method was accomplished in ABI 3500xl genetic analyzer followed by further analysis by Basic Local Alignment Search Tool. The sequencing result obtained after the process was submitted to the GenBank National Center for Biotechnology Information database. Phylogenetic tree was constructed using neighbour-joining method with a bootstrap value of 100 in MEGA version 11.0 (Tamura et al., 2021).

2.2 Estimation of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of PGT was checked to estimate the maximum concentration of PGT tolerated by the fungus. The assay was performed in minimal medium (pH 6.8) spiked with PGT. Series of flasks containing M1 medium were spiked with increasing concentration of PGT (0-250 mg/L), inoculated with 1 mL fungal spore suspension and incubated at 120 rpm for 5-7 days. After incubation, the concentration showing absolute inhibition of mycelial growth was noted as minimum inhibitory concentration (Jaowiecki et al., 2019).

2.3 Growth of the fungal isolate in the presence of PGT

The analysis of mycelial growth curve was compared in presence and absence of the PGT in two distinct setups: (i) first series of 7 flasks contained with 100 mL of PDB in each flask and 1 mL of fungal spore suspension was added and (ii) second set of 7 flasks comprised of 100 mL PDB in each flask, 1 mL of fungal spore suspension, and PGT (100 mg/L) [PDB^R]. These flasks were incubated at 120 rpm in a rotary shaker. Dry weight of the mycelium was measured and compared for growth curve analysis after every 24 h for 7 days (Chatterjee and Abraham, 2019).

2.4 Biotransformation experimental analysis in liquid medium

The ability of the isolate to breakdown PGT into non-toxic metabolite was carried out in PDB^R medium incubated at 28±2°C at 120 rpm. Degradation capacity of the fungal isolate was tested for 7 days in PDB^R. The dose of inoculation in the medium was maintained at 1% (v/v) spore suspension. The PGT residual concentration in culture was measured after every 24 h. Control setup was maintained without addition of fungal isolate to aid in determining the concentration variation due to hydrolysis. Since, minute changes in concentration might happen due to abiotic conditions, the concentration of PGT was also analysed regularly along with the test sample. The tests were performed in triplicates (Shi et al., 2021). The equation used to determine the biodegradation rate in the liquid medium is as follows:

$$Rate\ of\ Biotransformation\ (\%) = \frac{CC_t - TC_t}{CC_t} \times 100\%$$

In the above equation, CC_t denotes the residual PGT concentration in the control setup at *t* day (time), TC_t denotes the residual PGT concentration in the test setup inoculated with the fungal isolate measured during the same time *t*.

2.5 Estimation of progesterone concentrations by analytical analysis

2.5.1 Extraction of the samples by solid phase extraction

The extraction of PGT residual contents in the sample was carried out using 1:1 (v/v) mixture of methanol and water. 1 mL of filtered and centrifuged sample was allowed to pass through the cartridge followed by washing of cartridges. The eluted samples after the extraction were used for HPLC (Marta et al., 2010; Venier et al., 2021).

2.5.2 High performance liquid chromatography

PGT residual content was analyzed by HPLC (Young Lin, Acme 9000 South Korea) with C18 reversed phase column. HPLC grade methanol and water at a ratio of 70:30 v/v containing 0.1% formic acid was used as mobile phase. The flow rate of the mobile phase was maintained at 1 mL/min. 20 μL sample was injected and retention time was recorded maintaining a flow rate of 300 μL/min.

2.6 Chemical kinetics

The data attained after analysis of the results was applied in kinetics model to determine the rate contact of the degradation reaction. The time which was required to reduce the concentration of the drug by 50% was evaluated using linear equation from the regression between C_t-C_0 for zero order,

ln (C_t/C_0) for 1^{st} order, *ln* C_t for pseudo first order, *1/C* for 2^{nd} order and *t/*C_t for pseudo second order.

The following equations are used to derive the suitable model followed for the degradation:

- C_t-C_0 = kt (Zero order model)
- $C_t/C_0 = e^{-kt}$ (First order model)
- ln C_t = -kt + ln C_0 (Pseudo first order)
- 1/C = kt + 1/C_0 (Second Order)
- t/C_t = t/C_e + 1/kC_e ^{2}(Pseudo second order)

where, C_0 and C_e denotes the concentration of PGT in the liquid medium at "0^{th} time" and C_t and *C* indicates concentration of PGT at a particular time "t" (denoted by *t*). *k* represents the rate constant of the reaction.

2.7 Determination of end products after the transformation of progesterone

Successful breakdown of PGT was examined by gas chromatography mass spectroscopy (GC-MS) for determination of the final metabolites produced after the fungal action on the progesterone. Elite-5MS capillary GC column [30 m in lunch and 250 μm film thickness] (Perkin Elmer) was used for GC-MS analysis. The carrier gas (helium) was maintained at a rate of 1 mL/min. The full scan chromatogram obtained of the degraded sample was analyzed using the National Institute of Standards and Technology library database.

2.8 Microbial formulation for long term storage

Liquid bioformulation of the microbial isolate was prepared for further application in large scale. The liquid bioformulation was prepared in a ratio of 25:75 oil:water. Once microbial spore suspension was added to the oil:water suspension, constantly stirring was required using a magnetic stirrer. Upon formation of an emulsion, the formulation was stored in tightly closed air-tight bottles and checked for shelf life after regular intervals.

3 Results

3.1 Isolation, screening, and identification of PGT biodegrading fungal isolate

In the preliminary phase of the study, selective enrichment method was used to isolate PGT degrading fungi from hospital sewage soil sample. Three distinct PGT tolerant fungal strains were obtained. One isolate, among the three, was chosen for the further study for its maximum ability to tolerate PGT among the three. Suitable growth medium for the fungus was found to be potato dextrose medium. The isolate appeared to be dark brownish to black of PDA plates as shown in Figure 1(a). The reverse side of the plate was observed as grayish in color. Mycelial and spore characteristics observed using a light microscope indicated the fungus to be similar to *Aspergillus* sp. as shown in Figure 1(b). The taxonomic identification of the isolate was conducted using 18S rRNA gene sequencing and identified as *Aspergillus niger* designated as isolate NAAC with the accession ID OP485290. The obtained phylogenetic tree of the strain using neighbour joining method is shown in Figure 2.

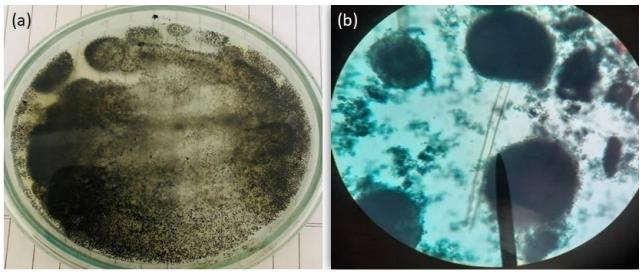

Figure 1. Colony morphology of Progesterone degrading fungus (a) on Potato Dextrose Agar plate and (b) spores observed under light microscope (100X).

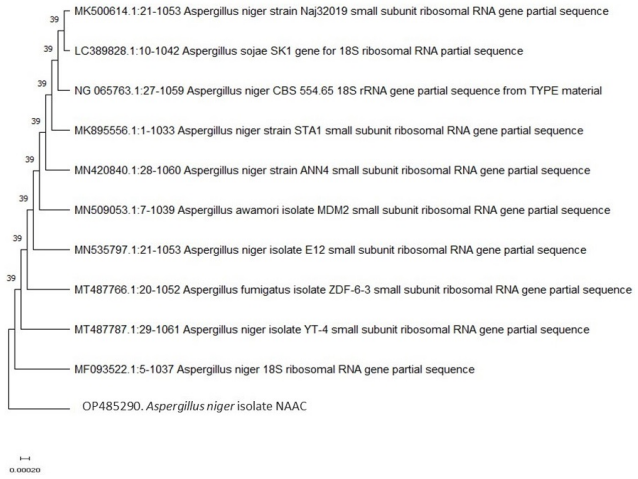

Figure 2. Phylogenetic tree of *Aspergillus niger* NAAC using neighbour joining method.

3.2 Microbial capacity of progesterone tolerance

Determination of the minimum inhibitory concentration was measured by estimating the complete inhibition of mycelial growth in the liquid medium. It was observed that the strain grew luxuriantly till 150 mg/L of PGT. The mycelial growth was observed to decline once the PGT concentration reached 250 mg/L.

3.3 Effect of PGT on growth of *Aspergillus niger* NAAC

The strain was allowed to grow in liquid medium with and without PGT. The mycelial growth obtained after each day indicated that the isolate was not suppressed by presence of PGT in the medium which is depicted in Figure 3.

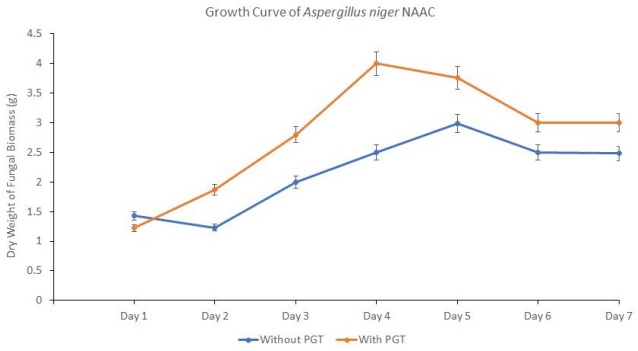

Figure 3. Growth curve of *Aspergillus niger* NAAC.

3.4 Biodegradation of PGT by *Aspergillus niger* NAAC

The removal of PGT in the aqueous medium was studied with the help of HPLC. During the study, it was analysed that the isolate was able to degrade 97% PGT at 100 mg/L concentration in a span of 3 days as represented in Figure 4.

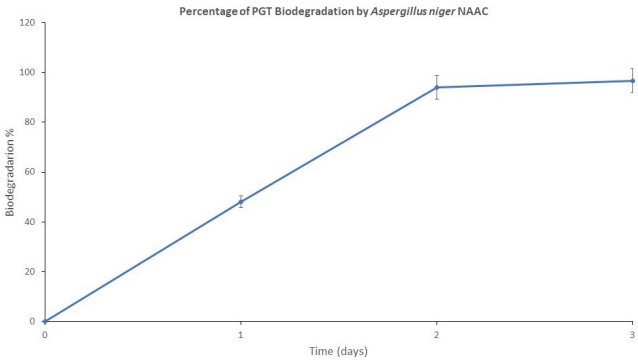

Figure 4. Percentage of Degradation of Progesterone by *Aspergillus niger* NAAC.

The metabolites produced after completion of degradation of the PGT were identified using GC-MS (chromatogram shown in Figure 5). The final end product was interpreted to be non-toxic in nature.

3.5 Kinetics analysis of biodegradation of progesterone

The results obtained from the biodegradation studies showed no significant elimination of PGT due to abiotic changes and thus the overall loss of the PGT in the medium was considered to be microbial activity. The process indicated that biodegradation of PGT was fit using pseudo first order kinetic model as graphically represented in Figure 6.

3.6 Microbial formulation

The low-cost liquid formulation of *Aspergillus niger* NAAC was prepared using used vegetable cooking oil (Figure 7(a). The shelf life and the rate of microbial contamination in the

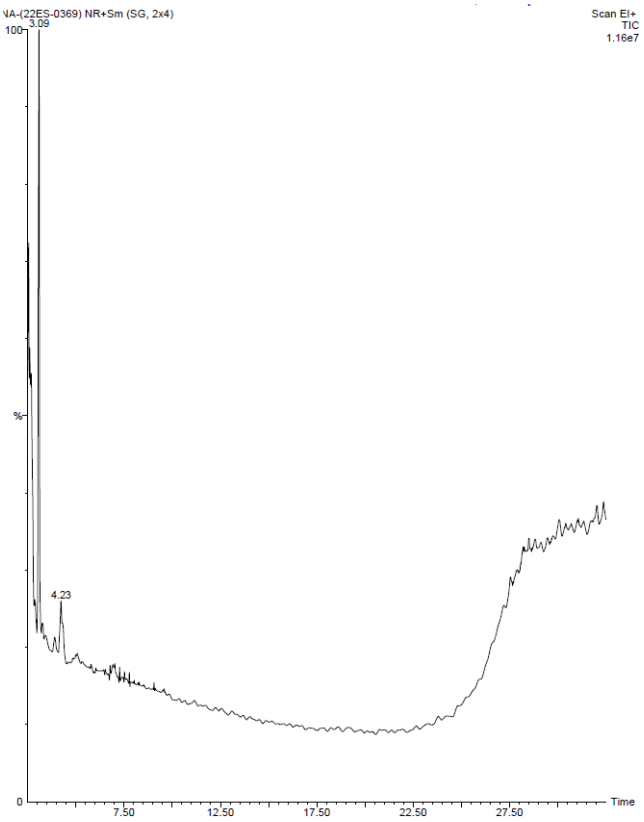

Figure 5. GC-MS chromatogram of the degraded sample obtained after fungal interaction.

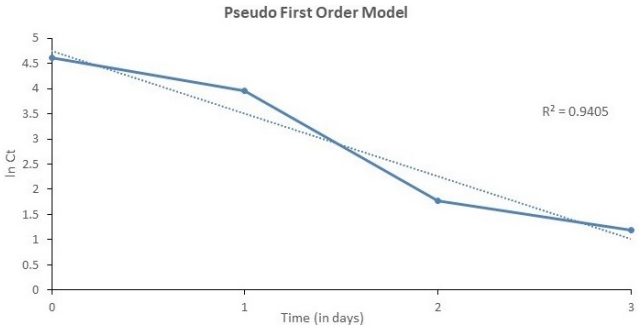

Figure 6. Pseudo First Order Kinetics fit of Biodegradation Process.

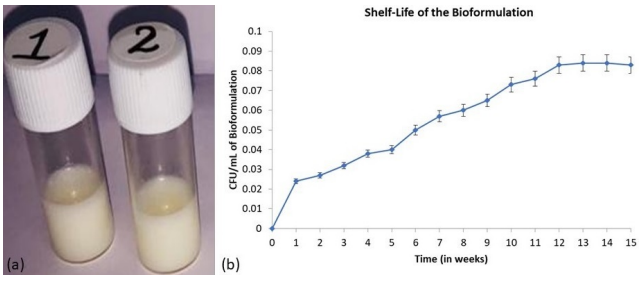

Figure 7. (a) Bioformulation of *Aspergillus niger* NAAC and (b) Viability of the fungal spores in the bioformulation.

formulation was assessed for 15 weeks at room temperature. The Figure 7(b) shows the viability of the isolate to be consistent during the period of study. The rate of contamination was found below the limit of detection which supports the efficacy of the formulation for long term usage. The analysis of shelf life was experimented in five trials for better evaluation of the formulated product.

4 Discussion

The study involved application of fungal strain *Aspergillus niger* isolate NAAC in biotransformation of progesterone in laboratory scale. The strain isolated from soil sample was identified using 18S rRNA sequencing. Colony morphology and preliminary observation under light microscope indicated the fungus to be *Aspergillus*. The phylogenetic tree of the identified strain was constructed using neighbour joining method (Tamura et al., 2021). The fungus was able to grow until 250 mg/L PGT concentration in the medium. In earlier experimental studies, *Rhodococcus* sp. was reported to show maximum growth in medium spiked with 500 μg/mL PGT (Yu et al., 2018). With time, the fungus could get acclimatize to the changes occurred by PGT in the medium and showed enhanced mycelial growth which confirmed that the organism was able utilize PGT as their carbon and energy source. *Talaromyces* sp., *Penicillium citrinum* H7 and *Aspergillus nidulans* VKPM F-1069 also effectively showed increased biomass growth when inoculated in PGT spiked medium as mentioned in earlier researches (Savinova et al., 2019; Dos Santos et al., 2022).

In liquid systems, effect of abiotic mechanism, such as, hydrolysis plays a crucial role in degradation. The overall mineralization of PGT thus attributes to the combined effect of hydrolysis and fungal isolate NAAC. However, during the biodegradation experimental studies in aqueous medium, it was observed that the PGT showed extreme stability towards abiotic stress and thus hydrolysis activity was negligible. Previous reports of bacterial degradation (*Rhodococcus* sp.) of PGT claimed 99% removal of the contaminant (Yu et al., 2018). Cyanobacteria and freshwater microalgae convert progesterone into androgens by diversified metabolic reaction pathways (Peng et al., 2014). Studies have revealed that androgens, such as, 1,4-pregnadiene-3,20-dione, androstenedione-3,20-allpregnanedione are produced when progesterone is treated by microalgae (Ojoghoro et al., 2017). However, the problem still remains to an extent since further breakdown of androgen are required in order to eliminate the complete toxicity of the steroid.

Considering the application of fungal strains in removal of PGT, the best claimed result till date was achieved by using *Penicillium citrinum* H7 which could remove around 90% of PGT (Dos Santos et al., 2022). The particular strain in this study, *Aspergillus niger* NAAC, thus, can be claimed as a more efficient agent for removal of the PGT from surrounding environment when compared with the previous published reports. The isolate could reduce the C4-C5 double bond of the steroid compound followed by oxidation of C-17. The predicted mechanism of breakdown of the PGT might be initial oxidation of C-3 followed by C-17 oxidation. Further, the metabolic compound loses four hydrogen atoms accompanied by dehydrogenation of the steroid backbone. Upon dehydrogenation, the compound undergoes hydroxylation and undergoes cleavage followed by microbial oxidation of C-4 and C-5. Finally, the C ring is converted into a molecule acid by oxidation-reduction reaction (Yu et al., 2018). The conversion process includes hydroxylation, hydrogenation, dehydrogenation and side-chain breakdown (Safiarian et al., 2021; Liu et al., 2020). Studies revealed that PGT is first transformed to hydroxyprogesterone which further undergoes side chain breakdown and dehydrogenation (Sangster et al., 2016). The final end product is non-toxic in nature and thus it can be claimed that microbial breakdown of PGT can lead to removal of the pollutant. The breakdown of the progesterone by fungal cultures is cost effective and flexible as compared to the conventional methods of progesterone elimination (Misra et al., 2021).

Bioformulation is an effective way to maintain the viability of microorganisms for a long period of time. The preparation of bioformulation aids in smooth transportation and administration of the effective microbial strain for the remediation treatment (Chakraborty et al., 2022). Application of bioformulation in remediation techniques also reduce the cost of the process. The cultivation of microbial species is not repeated for every cycle of the process if maintained in a bioformulation form. Waste vegetable oil are considered as non-edible feedstock after utilization in cooking and thus their reuse in production of formulation would reduce the chances of carefree disposal of these oils in environment which further becomes a non-biodegradable pollutant. Application of waste vegetable oil in preparation of bioformulation opens the chances of considering them as burden free feedstock. The waste vegetable oil serves as an inert material for storing the fungal spores and enhancing their shelf life for long term application (Fernandes et al., 2019; Bejarano and Puopolo, 2020).

5 Conclusion

During the experimental processes, the isolate showed high efficiency in breakdown of the steroid and production of non-toxic end-product. The low-cost liquid bioformulation was developed following affordable cost-effective processes and can be further used in large scale experimental setups. To our best knowledge, this is the first study reporting the production of bioformulation using a progesterone degrading microbial strain. Thus, the results obtained confirms the isolate NAAC to be a potential microbe for removal of the drug and further investigation might be carried out for field scale studies. The application in field trials might be useful in attributing to sustainable management of water (SDG 6 of United Nations)

since most of the progesterone contamination is observed in surface waters which makes the water non-consumable along with affecting the animal lives.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Author Contributions

The concept of the work and protocols was framed by Dr. Ankita Chatterjee. The execution of the work was carried out by Ms. Nida Afreen. The manuscript was drafted by Dr. Ankita Chatterjee.

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