

ORIGINAL ARTICLE

Biological Oxidation of Testosterone by *Rhizopus stolonifera* A microbial Approach to 11-Ketotestosterone Production and Its Degradation Due to Water Pollution Leading to Restriction of Fish Population

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ABSTRACT

11-Ketotestosterone (11-KT) is an important androgenic steroid hormone in fishes, playing a major role in reproductive functions and behaviour. This study determines the way of the biological oxidation of testosterone by *Rhizopus stolonifera*, fungal species for the synthesis of 11-KT and investigates its degradation under different water samples collected from different locations. The stability of 11-KT in sewage, tap water, lake water, and distilled water was analysed using spectrophotometry and FeCl₃ colorimetric testing. The degradation of 11-KT due to pollutants in aquatic environments is discussed in relation to fish pheromone disruption, potentially leading to reduced reproduction and declining in fish populations. By bridging the gap between aquatic pollution and fish pheromone communication, this study contributes to the broader discussion on environmental sustainability and its conservation. Understanding how pollutants affect fish reproductive behaviour is essential for developing strategies to mitigate the impacts of water contamination on freshwater ecosystems. Regulatory policies should emphasize reducing chemical runoff and wastewater discharge to preserve the integrity of aquatic signalling mechanisms essential for fish population stability. This study altogether focuses in maintaining the ecological balance by demonstrating the degradation of fish pheromone like 11Kt. Interrelation between environment chemistry and aquatic life is concluded under this research. So that researchers and policymakers took step to conserve the resources and to make sure about protection of freshwater habitats and the species dependent on it. Policies should be made to minimize the waste water discharge and chemical runoff to prevent the chemical signal blockage in aquatic life.

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INTRODUCTION

11-Ketotestosterone is a crucial androgen responsible for sexual development and reproductive behaviour in fishes. Environmental pollutants, including industrial waste and agricultural runoff and other major pollutants and hazards contribute to the degradation of 11-KT, leading to endocrine disruption in aquatic organisms. This study focuses on the microbial oxidation of testosterone by *Rhizopus stolonifera* [2] to produce 11-KT and evaluates its stability in different water matrices [4][5]. Understanding its degradation mechanisms is essential for clarify the impact of pollution on fish populations [49]. There are lot of researches have been done on biotransformation of many compounds. Some Fungal species have ability to oxidize this compound due to some enzymes present in their species. *Rhizopus stolonifer*, a fungal species commonly known as Black bread mold contain Fungal monooxygenase which oxidize testosterone at its 11-C position of ring and convert it into 11-beta-hydroxytestosterone. Later on that 11-beta-hydroxytestosterone further converted to 11- Keto-testosterone. 11-KT is a compound that is naturally present in some fish act as Sexual pheromone which attract the mating partner. Due to the artificial biotransformation, we can study various aspects of this

hormone rather than extracting it naturally. Biotransformation of testosterone is previously studied and documented [62]. But its 11- C position oxidation is being study. Main motive of this research is to get a biosynthesized 11-KT by fungal oxidation and demonstration of enzymatic activity of *Rhizopus* species. Because it is a fish androgen its general reaction with different water samples will showcase the fish-to-fish pheromone communication obstructs due to various environmental pollutants present in different sites. 11-Ketotestosterone (11-KT) is a potent androgen found in various aquatic organisms [50]. Its role in fish reproduction is vast as it is an androgenic factor [59]. The stability of 11-KT varies depending on pH, temperature, and organic matter content is said. [45]. The degradation of 11-KT in sewage and agricultural water sources highlights the potential ecological risks associated with pollution, particularly in urban and rural water systems. Fish populations that rely on chemical signalling for mating and territorial behaviour may experience disrupted reproductive cycles, ultimately leading to reduced population viability. TLC analysis of the byproduct of hormone can detect the endocrine disruptive products which are the result of chemical degradation. In teleost fishes, 11-ketotestosterone (11-KT) plays crucial role in secondary sexual characteristics, reproductive behaviour and aggression. In some species like goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*), and tilapia (*Oreochromis spp.*), 11-KT also functions as a pheromone that help in communication, mediating social and mating interactions [61] [60] Chemical communication is essential for fish reproduction, with pheromones playing a major role in mate selection and reproductive success. However, pollution, including contaminants from wastewater, agricultural runoff, and industrial discharge [31], may alter the stability and bioavailability of 11-KT [1], thereby disrupting chemical communication in fish populations. Water pollution has become a very major environmental concern, with anthropogenic activities [28] and our day-to-day life introducing a range of pollutants into aquatic ecosystems leading to pollution. Chemical pollutants, such as pesticides, heavy metals, hazardous waste, and endocrine-disrupting compounds, have been shown to interfere with hormonal pathways as well as communication signalling in fishes. Biological reactions and pH changes in waters bodies may also contribute to the degradation of bioactive molecules like 11-KT which leads to changes in its chemical form. Studies on steroid hormone stability in aquatic environments have mostly focused on oestrogens and androgens, with limited research on the persistence of 11-KT in different water -samples which is included in this research. It is said and known factor that many freshwater fish rely on 11-KT as a pheromone, its degradation in polluted environments may lead to reproductive impairments and population declines [21]. The role of water chemistry in determining the stability of 11-KT is largely unexplored, necessitating an investigation into how different water qualities affect its persistence is also. Understanding the factors that influence 11-KT degradation is crucial for assessing the impact of pollution on aquatic life and developing strategies to mitigate these effects.

MATERIAL AND METHODS

Fungal Biotransformation of Testosterone

- *Rhizopus stolonifera* was cultured in a suitable and maintained medium containing testosterone as substrate for the reaction.
- By the oxygenase enzyme oxidation occur by several day incubation period attempt and measuring the favourable environment.
- The incubation period is optimized for maximum conversion of testosterone to 11KT.
- Biomass is get transformed and fungal cell get maximum transformation and changes.
- The extraction of fungal biomass and culture media is done by using ethyl acetate by separation method of best yielding and extraction.
- The extract was purified and confirmed using thin layer chromatography (TLC) and spectroscopic technique.
- Obtained compound is stored for further analysis and tests.

Characterization of 11-KT

- Spectrophotometric analysis was conducted to confirm structural modifications.
- Spectrometric analysis is done to confirm structural modification as well as its stability.
- This will indicate the absorption wavelength of the compound compared with standard.
- Wavelength of spectrophotometry visualized the actual compound relativity. FeCl_3 colorimetric testing was used to detect the presence of ketone group in the compound.
- pH dependent stability was evaluated to determine degradation rates in different water samples.

Environmental Degradation Study

- 11KT was introduced into sewage, tape water, lake water, and distilled water.

- Collection of water is done with pH analysis showcasing the actual pH of water sample collected and comparing it with changes in it [6].
- Degradation was monitored using UV- visible spectroscopy over time.
- Effect of pollutants such as heavy metals, pesticides, environmental hazards and organic matter content on 11 KT were assessed.
- The ferric chloride analysis is done to determine the colour changes with respect to the time.

RESULTS

The results showcase significant degradation in polluted water sources, particularly in sewage and water bodies such as lake due to microbial activity, chemical contamination, and pH fluctuation which is ultimate effect of anthropogenic activity. Degradation is observed very high in sewage water which associated with the pollutants but also medium in lake water which is concerning.

Lake water was collected from Man-Sagar lake, Jaipur and the degradation showcase the lower water quality of the lake. These findings suggest that external pollutants accelerate the breakdown of hormones due may be their presence in body of fish and ultimately blockage of pheromones. This may lead to endocrine disruption and decrease in fish population.

- *Rhizopus stolonifera* successfully oxidized testosterone to 11-KT, confirmed by spectrophotometry and TLC.
- *Rhizopus stolonifera* successfully transformed testosterone to 11KT by oxidation, confirmed by spectrophotometry and TLC.
- Degradation was significantly higher in sewage and lake water due to microbial activity and pollutants.
- Spectroscopic analysis determined the time vs absorption curve which demonstrate the degradation of compound with time.
- The FeCl₃ test indicated a reduction in ketone functionality over the time, supporting oxidative degradation mechanism.
- Pollutants in aquatic system facilitated 11 KT breakdown, leading to reduced pheromone signals in fish population.

DISCUSSION

The biotransformation of testosterone by *Rhizopus stolonifera* provides an efficient method for 11KT production.

Testosterone (C₁₉H₂₈O₂₀)

↓
(Fungal monooxygenase, O₂)

11β-Hydroxy-testosterone (C₁₉H₃₀O₃)

↓
(11β-HSD Dehydrogenase, NAD⁺)

11-Ketotestosterone (C₁₉H₂₈O₃)

The rapid degradation of 11 KT in polluted water highlights the vulnerability of fish to environment contaminants.

Disruption of pheromone signals due to hormone degradation can lead to reduced reproductive success and declining fish populations. Further research is needed to explore mitigation strategies to preserve fish reproductive health in contaminated habitats

Table 1: Variation in pH of water samples containing 11 KT with respect to time

Water Sample	Initial pH	Variation in pH	Possible Explanation
Distilled water	≈ 7	No significant changes	No reactive ion present (control)
Tap water	7.39	Slight drop (7.0 to 7.10)	Chlorine oxidation
Sewage water	6.62	Significant drop (approx.5)	Microbial degradation and organic acid formation
Lake water	6.96	Moderate drop (approx.6)	Agriculture run off and minerals interaction with 11 KT

Changes in pH showcases the study linkage between water chemistry and 11-KT and detects its stability.

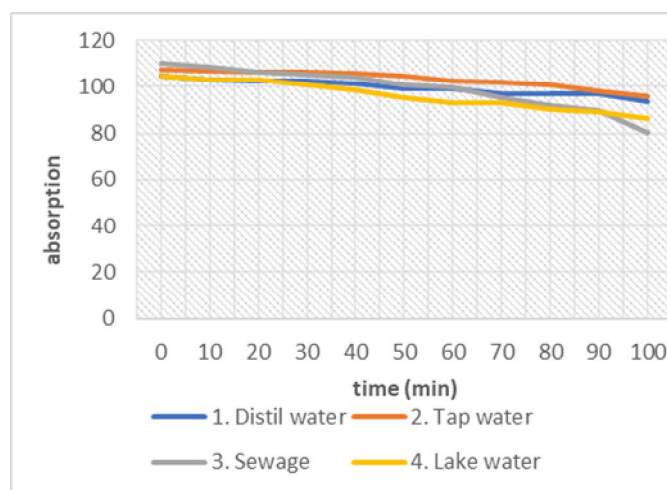
pH Testing (11- KT stability is influenced by pH fluctuation):

1. **Acidic conditions (pH < 6)** may lead to **hydrolysis** of the ketone group.
2. **Acidic condition (pH less than 7)** may lead to hydrolysis of the ketone group.
3. **Neutral to slightly alkaline (pH from 7 to 8)** is where 11 KT is most stable and degradation is very less.
4. **Highly alkaline conditions (pH more than 9)** can promote oxidation or structural modifications.

Table 2: FeCl₃ test of water samples containing 11-KT with respect to time

Water Sample	Colour Variation With Respect To Time	Possible Molecular Changes
Distilled Water	Stable yellow-brown colour	No degradation (control)
Tap Water	Gradual fading	Chlorine oxidation
Sewage Water	Rapid colour loss	Microbial degradation, organic interaction Chemical
Lake Water	Faster fading, turbidity	Chemical interaction (organic matter, minerals)

FeCl₃ testing determine the degradation of the 11KT with respect to time which is obtained in the results in above table. These results detected that rapid colour changes occur in sewage water meanwhile very less changes occur in the distilled water. Whoever also a faster fading occurs in lake water, which is result of pollutants present in the water resource. So that the chemical interaction takes place leading to change in structure of 11-KT.



Graph 1: Absorption versus time graph by spectrophotometric analysis of water samples containing 11-KT

Spectrophotometry

Above graph results showcases the degradation of the 11-KT absorption with respect to time. More degradation takes place in Sewage water due to degradation and chemical changes that occur in the Compound with respect to time. Secondly in lake water where the sample of lake water. Very lower changes are noticed in distil water and tap water which was initially hypothesized. Results showcase how water pollutants can change the nature of compound that plays crucial role in mitigation of fishes. Due to these changes in compounds fish communication is obstructed and lead to ultimate population decrease. Spectrophotometric analysis with different samples also suggests purity of chemical and its degradation under pollutant as a factor [22]. Aquatic life communicates with certain pheromones including the studied 11 KT so its degradation means disruption of the communication.

CONCLUSION

The microbial approach of oxidation of compounds is very relevant and cost-effective process biotransformation with the help of *Rhizopus stolonifera*, showcase the biotechnological approach for steroid oxidation of testosterone to 11KT. So that artificial synthesis is done without using harmful chemicals for oxidation. However, degradation of the 11-Ketotestosterone under polluted water condition poses a significant threat to aquatic ecosystem by impairing fish reproduction and its hormonal changes. This research address that environmental contamination is a factor to maintaining fish population and

biodiversity ultimately results in decline in the fish population. The large breakdown of 11 KT in sewage and other water samples shows that such pollutants like microbial contaminants, pesticides, and other pollutants may cause its breakdown. Since 11-KT is an essential chemical cue for reproduction success and mating in a majority of freshwater fishes, its breakdown may have extensive ecological implications. Disruption of pheromone communication caused by pollution in the environment can result in reduced reproductive efficiency among fish populations, eventually influencing population dynamics and health of the ecosystem. In the event that fish are not able to effectively sense reproductive signals, mating success will decrease, resulting in lower genetic diversity and possible collapse of population in high-pollution habitats. This is of particular concern for organisms that respond heavily to chemical cues for breeding and territory establishment. Furthermore, the results highlight the importance of having strict water quality regulations aimed at reducing discharge of pollutants that lowers the levels of vital bioactive compounds such as 11 KT. Improved wastewater treatment, management of industrial runoff, and tighter pollution control should be among the strategies put in place to reduce the adverse effects on aquatic ecosystems. It is recommended that future studies should aim at determining particular degradation pathways of 11KT as well as the breakdown products for possible endocrine disrupting activity. Further study can detect the actual chemical signalling changes due to which fish population can decline. The real agent behind the change in the population density is due to the pollutants in the water content which changes the chemical nature and causing the actual disruption of endocrinal hormone function.

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