**Effect of salinity on hyphal growth and antimicrobial metabolite production in two halophilic fungi**

**ABSTRACT:**

Halotolerant fungi possess the unique ability to survive and thrive in hypersaline environments, making them promising candidates for the production of bioactive secondary metabolites. This study investigated the effect of varying salinity levels (5–25% NaCl) on the growth, antimicrobial activity, and chemical profile of two halophilic fungal strains, Fusarium oxysporum RAS2 and Aspergillus niger RAS3, previously isolated from the Vellar estuary. Maximum hyphal growth for both strains was observed at 5% NaCl, whereas the highest antibacterial and antifungal activities were recorded at 20% NaCl. Ethyl acetate extracts from cultures grown at this concentration exhibited significant inhibition against Pseudomonas sp. and Candida albicans (F. oxysporum RAS2), and Pseudomonas sp. and Rhizopus sp. (A. niger RAS3). FTIR analysis revealed the presence of functional groups including alcohols, phenols, ketones, and amines, which are known to contribute to antimicrobial activity. These results suggest that salt-induced stress enhances the biosynthesis of potent antimicrobial compounds in halotolerant fungi, underscoring their potential as valuable sources of novel therapeutics.

**KEYWORDS:** Halophilic fungi, Fusarium oxysporum, Aspergillus niger, Crude extract, Antimicrobial activity

1. **INTRODUCTION**

Fungi found in hypersaline environments exhibit halophilic behavior distinct from that of most halophilic prokaryotes (Plemenitas et al., 2008; Ramesh et al., 2020; Anbuselvan et al., 2025). Among halotolerant fungi, key representatives include *Aspergillus niger*, *A. sydowii*, *Eurotiumamstelodami*, and *Penicilliumchrysogenum* (Gunde-Cimerman et al., 2009). The most abundant and well-adapted species is the black yeast *Hortaeawerneckii*, which naturally thrives under a wide range of fluctuating salt concentrations(Rani et al., 2013). Another example is *Wallemia ichthyophaga*, a true halophile that requires high salt concentrations for survival (Zalar et al., 2005). High sodium ion concentrations generally cause cell membrane damage due to osmotic imbalance (Plemenitas et al., 2014). However, halotolerant fungi counteract this stress by synthesizing and accumulating polyols such as glycerol, erythritol, arabitol, and mannitol. These polyols help maintain osmotic balance and prevent cellular damage (Gunde-Cimerman et al., 2018). Additionally, halophilic microorganisms are a potential source of novel bioactive compounds, as hypersaline conditions can activate otherwise silent genes involved in secondary metabolite biosynthesis (Koch, 1993; Méjanelle et al., 2001; Lu et al., 2008). Another adaptation observed in halotolerant fungi is melanin deposition on the cell wall, which reinforces the structure against osmotic shock, particularly in fungal hyphae exposed to constantly changing salinity (Elsayia et al., 2022). Elevated salt concentrations have also been shown to enhance the production of secondary metabolites in some halotolerant fungi, many of which exhibit antifungal and antibacterial activity (Panchal et al., 2022; Wingfield et al., 2023). Several species of halotolerant filamentous fungi are known to produce unique extrolites, including extracellular enzymes and organic acids (Bills et al., 2002; Margesin&Schinner, 2001; Behal, 2003).

For instance, the germination of conidia in *A. niger*, *A. terreus*, *A. flavus*, and *Emericellanidulans* is delayed in 10–15% NaCl, whereas moderate salinity levels (4–5%) stimulate growth(Ramaswamy&Krishnamurti, 1979). Similarly, at 3–5% NaCl, fungal growth and extrolite production are significantly enhanced(Masuma et al., 2001). *Eurotium* species produce various bioactive compounds such as echinulins, neoechinulins, and cryptoechinulins, while other fungi synthesize emodin, auroglaucin, physcion, dihydroauroglaucin, flavoglaucin, and tetrahydroauroglaucin (Butinar et al., 2005).Under mesophilic conditions, some fungal species also produce bioactive metabolites. For example, species of *Fusarium* have demonstrated antifungal (Evidente et al., 1994; Meca et al., 2010), antibacterial(Renner et al., 1998; Meca et al., 2010), anti-inflammatory (Renner et al., 2000), and cytotoxic(Belofsky et al., 1999) activities. Likewise, *Cladosporium* spp. have shown antibacterial (Judulco et al., 2001), antifungal, and cytotoxic properties (Shigemori et al., 2004), and *Aureobasidiumpullulans* has been reported to exhibit anticoagulant, antithrombotic, and antiviral potential(Chi et al., 2009).Our previous study demonstrated significant antibacterial activity in primary screenings of two halophilic fungi, *Fusariumoxysporum* RAS2 and *Aspergillusniger* RAS3 (Anbuselvan et al., 2025). The present study aims to optimize the culture conditions of these two strains to enhance bioactive compound production. Specifically, we empirically investigated the effects of salinity on the mycelial growth of *F. oxysporum* RAS2 and *A. niger* RAS3, as well as on their bioactivity and metabolite profiles.

1. **MATERIALS AND METHODS**

**2.1 Test Organisms:**

The fungal strains *F. oxysporum* RAS2 and *A. niger* RAS3, previously isolated from the Vellar estuary during our earlier study, were used for the present investigation (Fig.1)

**2.2 Hyphals Growth Assay:**

Potato Dextrose Broth (PDB) agar plates were prepared by supplementing with varying concentrations of sodium chloride (5%, 10%, 15%, 20%, and 25%). Wells were aseptically punched into the agar, and 10 µL of a 2-day-old fungal broth culture was inoculated into each well. The plates were incubated at 37°C for 24 hours. After incubation, the diameter of hyphal growth was measured to assess salt tolerance and growth efficiency.

**2.3 Mass Culture Preparation:**

To produce biomass for extraction, 150 mL of PDB medium containing different NaCl concentrations (5%, 10%, 15%, 20%, and 25%) was prepared. Each medium was inoculated separately with *F. oxysporum* RAS2 and *A. niger* RAS3. The cultures were incubated in a shaking incubator at 100 rpm for 4 days at 37°C.

**2.4 Extraction of Bioactive Compounds:**

After incubation, an equal volume of ethyl acetate was added to each broth culture and mixed in a shaking incubator at room temperature for 1 hour at 100rpm. After that, the cultures were filtered through Whatman No. 1 filter paper to separate the mixture of solvent and metabolite. The solvent was separated and concentrated using a rotary evaporator (IKV® RV10). The resulting semi-solid crude extract was re-dissolved in 5ml of ethyl acetate for further analysis.

**2.5 Antibacterial and Antifungal Activity Assay:**

The bioactivity of the extracts was tested against a panel of clinical bacterial and fungal pathogens. Bacterial strains included *Bacillus subtilis*, *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Pseudomonas* sp., *Salmonella paratyphi*, *Salmonella typhi*, *Shigella* sp., and *Vibrio harveyi*. Fungal strains included *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Candida albicans*, and *Rhizopus* sp. All strains were obtained from Rajah Muthiah Medical College (RMMC), Chidambaram.Muller-Hinton Agar (MHA) plates were prepared, and the pathogens were evenly swabbed onto the agar surface under sterile conditions. Wells were punched into the agar, and 100 µL of each fungal extract was introduced into the wells. Plates were incubated at 37°C for 24 hours, after which the zones of inhibition were measured. All assays were conducted in triplicate.

**2.6 Fourier Transform Infrared (FTIR) Spectroscopy**

The chemical composition of the crude extracts was analyzed using Fourier Transform Infrared (FTIR) spectroscopy. Spectra were recorded in the 650–4000 cm⁻¹ range using an Agilent Cary 630 FTIR spectrometer. Analyses were performed in triplicate at the Department of Chemistry, Annamalai University.

1. **RESULTS**

#### ****3.1 Effect of Salinity on Fungal Growth:****

The hyphal growth of F. oxysporum RAS2 and A. niger RAS3 was highest at 5% sodium chloride concentration. As salinity increased beyond this level, a gradual decline in mycelial growth was observed. The results indicate that both strains exhibit optimal growth under mild saline conditions, with a noticeable reduction at higher salt concentrations. Detailed measurements of hyphal growth across various NaCl concentrations are presented in **Table 1.**

#### ****3.2 Effect of Salinity on Antibacterial Activity:****

The antibacterial activity of ethyl acetate extracts from F. oxysporum RAS2 and A. niger RAS3 cultured at different salinity levels is summarized in **Tables 2**and **3.** Extracts from cultures grown in 20% NaCl exhibited the strongest antibacterial effects, particularly against Pseudomonas sp. The extract from F. oxysporum RAS2 (20%) showed a maximum zone of inhibition of **34 ± 0 mm**, while A. niger RAS3 (20%) demonstrated a zone of **42 ± 0.1 mm** against the same bacterium. In contrast, extracts from 5% and 25% NaCl cultures exhibited minimal antibacterial activity.

#### ****3.3 Effect of Salinity on Antifungal Activity:****

The antifungal potential of the crude extracts is shown in **Tables 4** and **5**. The highest antifungal activity was observed in extracts from fungi cultured in 20% NaCl. Specifically, F. oxysporum RAS2 exhibited a maximum zone of inhibition of **35 ± 0.6 mm** against Candida albicans, while A. niger RAS3 showed the highest inhibition (**45 ± 0.2 mm**) against A. niger. Similar to the antibacterial results, antifungal activity was significantly reduced in the extracts obtained from 5% and 25% NaCl cultures.

#### ****3.4 Effect of Salinity on Functional Group Profile:****

FTIR analysis of crude extracts from F. oxysporum RAS2 and A. niger RAS3 cultured under different salinity conditions revealed the presence of various functional groups, as shown in **Tables 6** and **7**. The transmittance graph of the crude extract of F. oxysporum RAS2 and A. niger RAS3 cultured in 20% NaCl concentration is given in Fig.2. Key functional groups detected included alcohols, phenols, alkynes, amines, ketones, and aliphatic compounds. These findings suggest the biosynthesis of structurally diverse metabolites under hypersaline conditions.

1. **DISCUSSION**

Physical parameters such as incubation time, temperature, pH, and salinity significantly influence the production of secondary metabolites in fungi, as supported by several previous studies (Suzuki et al., 1997; Rizk et al., 2007; Nishihara et al., 2001). Miao et al. (2006) reported that the marine-derived fungus *Arthrinium cf. saccharicola* showed optimal growth in 0 ppt salinity, whereas the highest antimicrobial activity was observed at 34 ppt against *Vibrio vulnificus* and *Pseudoalteromonas sspongiae*. In our study, *F. oxysporum* RAS2 and *A. niger* RAS3 exhibited maximum hyphal growth at 5% sodium chloride, while the highest antibacterial and antifungal activity was recorded in the extracts obtained from cultures grown in 20% NaCl. This pattern suggests that these fungal strains may have originated from terrestrial or freshwater environments, and that the production of antimicrobial metabolites was enhanced as a stress response to elevated salinity.Halotolerant fungi are known to evolve distinct metabolic strategies to cope with fluctuating saline environments. Some marine-derived fungal strains demonstrate increased growth under higher saline conditions, while their antimicrobial activity peaks in media containing 25–50% seawater (Masuma et al., 2001; Bugni and Ireland, 2004). Masuma et al. (2001) observed that certain marine fungal isolates, capable of thriving in high-salt conditions, also exhibited rapid growth in media containing 4% sodium chloride, implying that such species have adapted to their saline niches and may produce more antimicrobial metabolites as a survival mechanism. These adaptations, however, remain largely untapped in biotechnology.

In the current study, both *F. oxysporum* RAS2 and *A. niger* RAS3 demonstrated the highest antimicrobial potential when cultured in 20% NaCl. Specifically, the crude extract from *F. oxysporum* RAS2 at this concentration produced a maximum inhibition zone of 34 ± 0.8 mm against *Pseudomonas* sp. and 35 ± 0.7 mm against *Candida albicans*. Similarly, *A. niger* RAS3 extract inhibited *Pseudomonas* sp. with a zone of 42 ± 0.9 mm and *Rhizopus* sp. with 45 ± 0.8 mm. These results are in line with earlier reports highlighting the production of novel bioactive compounds such as nafuredin, phenochalasin(Tomoda et al., 1999), and roselipin(Omura et al., 1999) from marine fungi. Such findings reinforce the potential of marine and halotolerant fungi as promising sources of novel therapeutic agents.

FTIR analysis has been widely used to identify functional groups responsible for biological activities. Radhika and Mohaideen (2015) demonstrated the utility of IR spectroscopy in determining bioactive components, particularly by identifying peaks within the 4000–3400 cm⁻¹ range associated with alcohols and acids. These functional groups are often correlated with antimicrobial efficacy (Dalal et al., 2012). In our study, FTIR spectra of fungal extracts showed prominent peaks at 3444 cm⁻¹ and 3362 cm⁻¹, indicating the presence of alcohol and phenol groups. These compounds are known contributors to antibacterial activity (Sun et al., 2018), consistent with the antimicrobial performance observed. Additional peaks at 2981 cm⁻¹ (alkyne group), 1632 cm⁻¹ (N-H bend of amines), and 1714 cm⁻¹ (ketones and saturated aliphatic groups) further confirm the complex chemical profile of the extracts obtained from cultures grown in 20% NaCl, and highlight their potential as antimicrobial agents.

### ****CONCLUSION****

The present study highlights the influence of salinity on the growth and bioactive compound production of two halotolerant fungi, F. oxysporum RAS2 and A. niger RAS3. While optimal hyphal growth was observed at 5% NaCl concentration, the maximum antibacterial and antifungal activities were recorded at 20% salinity. This suggests that elevated salt levels act as a stressor, triggering the enhanced synthesis of antimicrobial secondary metabolites. FTIR analysis of the crude extracts revealed the presence of functional groups such as alcohols, phenols, ketones, amines, and alkynes, which are known to contribute to antimicrobial activity. These findings emphasize the potential of halotolerant fungi from hypersaline environments as promising sources for novel antimicrobial agents. Further studies on the purification and structural characterization of these bioactive compounds could lead to the development of new therapeutic drugs.

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

Author(s) hereby declared that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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**Table 1. Effect of salinity on fungal growth**

|  |  |  |
| --- | --- | --- |
| **Sl. No** | **Strains** | **Growth (mm)** |
| **5%** | **10%** | **15%** | **20%** | **25%** |
| 1. | *F. oxysporum* RAS2 | 30 | 24 | 19 | 15 | 8 |
| 2. | *A. niger* RAS3 | 34 | 30 | 26 | 22 | 13 |

**Table 2.Effect of salinity on antibacterial activity by *F. oxysporum* RAS2**

|  |  |  |
| --- | --- | --- |
| **Sl. No** | **Pathogen** | **Zone of inhibition (mm)** |
| **5% salinity** | **10% salinity** | **15% salinity** | **20% salinity** | **25% salinity** |
| 1. | *B. subtilis* | 13±0.5 | 23±0.5 | 26±0.7 | 30±0.7 | 10±0.5 |
| 2. | *E. coli* | 09±0.4 | 24±0.7 | 28±0.8 | 32±0.8 | 06±0.7 |
| 3. | *Klebsilla sp.* | 04±0.2 | 10±0.4 | 20±0.6 | 25±0.6 | 03±0.5 |
| 4. | *Proteus sp.* | 20±0.7 | 24±0.7 | 28±0.9 | 32±0.8 | 11±0.7 |
| 5. | *Pseudomonas sp.* | 18±0.3 | 22±0.4 | 27±0.7 | 34±0.8 | 09±0.4 |
| 6. | *S. paratyphi* | 25±0.7 | 28±0.7 | 29±0.7 | 30±0.7 | 12±0.7 |
| 7. | *S. typhi* | 21±0.4 | 23±0.5 | 25±0.6 | 28±0.6 | 11±0.8 |
| 8. | *Shigella sp.* | 09±0.2 | 16±0.1 | 20±0.6 | 21±0.5 | 05±0.6 |
| 9. | *V. harveyi* | 19±0.3 | 22±0.4 | 28±0.8 | 32±0.8 | 09±0.7 |

**Table 3.Effect of salinity on antibacterial activity by *A. niger* RAS3**

|  |  |  |
| --- | --- | --- |
| **Sl. No** | **Pathogen** | **Zone of inhibition (mm)** |
| **5% salinity** | **10% salinity** | **15% salinity** | **20% salinity** | **25% salinity** |
| 1. | *B.subtilis* | 17±0.2 | 28±0.6 | 30±0.7 | 39±0.8 | 09±0.6 |
| 2. | *E. coli* | 18±0.3 | 28±0.7 | 32±0.8 | 36±0.7 | 08±0.8 |
| 3. | *Klebsilla sp.* | 16±0.2 | 22±0.5 | 28±0.6 | 36±0.7 | 05±0.6 |
| 4. | *Proteus sp.* | 19±0.3 | 25±0.5 | 32±0.7 | 36±0.6 | 07±0.7 |
| 5. | *Pseudomonas sp.* | 29±0.6 | 34±0.8 | 36±0.7 | 42±0.9 | 11±0.9 |
| 6. | *S.paratyphi* | 26±0.6 | 29±0.7 | 32±0.7 | 38±0.6 | 09±0.8 |
| 7. | *S.typhi* | 16±0.3 | 24±0.5 | 27±0.5 | 41±0.8 | 07±0.6 |
| 8. | *Shigella sp.* | 09±0.3 | 26±0.5 | 32±0.7 | 40±0.8 | 04±0.4 |
| 9. | *V.harveyi* | 21±0.5 | 30±0.7 | 33±0.8 | 38±0.7 | 11±0.9 |

**Table 4.Effect of salinity on antifungal activity by *F. oxysporum* RAS2**

|  |  |  |
| --- | --- | --- |
| **Sl. No** | **Pathogen** | **Zone of inhibition (mm)** |
| **5% salinity** | **10% salinity** | **15% salinity** | **20% salinity** | **25% salinity** |
| 1. | *A.flavus* | 25±0.5 | 28±0.5 | 30±0.6 | 31±0.7 | 12±0.8 |
| 2. | *A.fumigatus* | 19±0.4 | 24±0.5 | 28±0.5 | 30±0.6 | 09±0.5 |
| 3. | *A.niger* | 20±0.4 | 26±0.5 | 29±0.5 | 33±0.8 | 11±0.7 |
| 4. | *Rhizopus sp.* | 09±0.3 | 21±0.4 | 26±0.4 | 27±0.5 | 04±0.6 |
| 5. | *C. albicans* | 21±0.4 | 27±0.5 | 31±0.6 | 35±0.7 | 09±0.4 |

**Table 5.Effect of salinity on antifungal activity by *A. niger* RAS3**

|  |  |  |
| --- | --- | --- |
| **Sl. No** | **Pathogen** | **Zone of inhibition (mm)** |
| **5% salinity** | **10% salinity** | **15% salinity** | **20% salinity** | **25% salinity** |
| 1. | *A.flavus* | 22±0.4 | 31±0.7 | 34±0.6 | 38±0.6 | 09±0.7 |
| 2. | *A.fumigatus* | 22±0.3 | 36±0.8 | 37±0.6 | 39±0.6 | 11±0.5 |
| 3. | *A.niger* | 29±0.7 | 34±0.7 | 39±0.7 | 42±0.8 | 10±0.8 |
| 4. | *Rhizopus sp.* | 30±0.6 | 32±0.6 | 38±0.6 | 45±0.8 | 12±0.6 |
| 5. | *C. albicans* | 31±0.7 | 35±0.7 | 39±0.7 | 41±0.7 | 14±0.9 |

**Table 6.Effect of salinity on functional group profile in *F. oxysporum* RAS2**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sl.No.** | **Frequency range** **(cm-1)** | **5% salinity** | **10% salinity** | **15% salinity** | **20% salinity** |
| 1. | 3444 | - | O-H stretch of alcohol, phenol | - | - |
| 2. | 3362 | - | - | - | O-H stretch of alcohol, phenol |
| 3. | 2981 | C-H stretch of alkenes | C-H stretch of alkenes | C-H stretch of alkenes | - |
| 4. | 2109 | - | - | - | -C≡C- stretch of alkynes |
| 5. | 1736 | C=O stretch of carbonyls, carboxylic acids | - | C=O stretch of carbonyls, carboxylic acids | - |
| 6. | 1714 | - | C=O stretch of ketones, saturated, aliphatic | - | - |
| 7. | 1632 | - | - | - | N-H bend 1º amines |
| 8. | 1371 | C-H rock alkanes | C-H rock alkanes | C-H rock alkanes | - |
| 9. | 1237 | C-O stretch of alcohols, carboxylic acids, esters, and ethers | C-O stretch of alcohols, carboxylic acids, esters, and ethers | C-O stretch of alcohols, carboxylic acids, esters, and ethers | - |
| 10. | 1043 | C-N stretch of aliphatic amines | C-N stretch of aliphatic amines | C-N stretch of aliphatic amines | - |

**Table 7. Effect of salinity on functional group profile in *A. niger* RAS3**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sl.No** | **Frequency range** **(cm-1)** | **5% salinity** | **10% salinity** | **15% salinity** | **20% salinity** |
| 1. | 3541 | O-H stretch of alcohol, phenol | - | - | - |
| 2. | 3444 | - | - | - | O-H stretch of alcohol, phenol |
| 3. | 3414 | - | - | O-H stretch of alcohol, phenol | - |
| 4. | 2981 | C-H stretch of alkenes | C-H stretch of alkenes | C-H stretch of alkenes | C-H stretch of alkenes |
| 5. | 1736 | C=O stretch of carbonyls, carboxylic acids | - | - | - |
| 6. | 1729 | - | C=O stretch of ἀ, B,- unsaturated esters | - | - |
| 7. | 1714 | - | - | C=O stretch of ketones, saturated aliphatic | C=O stretch of ketones, saturated aliphatic |
| 8. | 1371 | C-H rock alkanes | C-H rock alkanes | C-H rock alkanes | C-H rock alkanes |
| 9. | 1244 | - | - | C-O stretch of alcohols, carboxylic acids, esters and ethers | - |
| 10. | 1237 | C-O stretch of alcohols, carboxylic acids, esters, and ethers | C-O stretch of alcohols, carboxylic acids, esters, and ethers | - | - |
| 11. | 1043 | C-N stretch of aliphatic amines | C-N stretch of aliphatic amines | C-N stretch of aliphatic amines | - |

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**Fig.1. Pure culture (A) Fusarium oxysporum RAS2; (B) Aspergillus niger RAS3**

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**Fig. 2. FTIR spectrum of crude extract of fungi cultured in 20% NaCl concentration (A) Fusarium oxysporum RAS2; (B) Aspergillus niger RAS3**