

STRAIN IMPROVEMENT OF *ASPERGILLUS TUBINGENSIS* FOR ENHANCED POLYURETHANE BIODEGRADATION THROUGH SEQUENTIAL UV AND EMS MUTAGENESIS

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Abstract

Polyurethane (PU) is one of the most widely used synthetic polymers globally, yet its remarkable chemical durability renders it essentially non-biodegradable under natural conditions, contributing substantially to the plastic pollution crisis. Bioremediation using filamentous fungi offers a promising eco-friendly alternative, with *Aspergillus tubingensis* having demonstrated notable capacity for polyester-polyurethane degradation through secretion of hydrolytic enzymes. However, the degradation rates of wild-type strains remain insufficient for practical application. This study employed sequential ultraviolet (UV) and ethyl methane sulfonate (EMS) mutagenesis to develop hyper-producing mutant strains of *A. tubingensis* with significantly enhanced polyurethane biodegradation capability. UV exposure at 254 nm for 15 minutes (achieving 95% kill rate) followed by 1% EMS treatment for 150 minutes (90% kill rate) was applied to generate a library of approximately 5,000 mutants. Primary screening on polyurethane agar plates identified 47 mutants (0.94%) with degradation index (DI) values exceeding 2.0, compared to 1.30 for the wild-type. Secondary screening in liquid mineral salt medium over 28 days identified five hyper-producing mutants (AT-M1 to AT-M5) achieving 34.2–38.4% polyurethane weight loss, representing 1.83–2.05-fold enhancement over the wild-type (18.7%). FTIR-ATR analysis confirmed enhanced ester bond cleavage, with mutant AT-M1 showing 46.7% reduction in the ester carbonyl peak (1728 cm⁻¹) versus 22.8% for the wild-type. Scanning electron microscopy (SEM) revealed extensive surface erosion, deep crack formation, and hyphal penetration in mutant-treated films. Enzyme assays demonstrated significantly elevated esterase specific activity (44.2 U/mg protein for AT-M1 vs. 25.8 U/mg for wild-type; 1.71-fold) and protease activity (1.55-fold), with strong positive correlations between esterase activity and weight loss ($R^2 = 0.96$). All five mutants retained >95% of their degradation capacity after five subcultures, confirming genetic stability. These results establish sequential mutagenesis as an effective strategy for enhancing fungal polyurethane biodegradation, with mutant AT-M1 representing a promising biocatalyst for PU waste bioremediation applications.

1. Introduction

The twentieth century witnessed an unprecedented revolution in materials science, with the development and widespread adoption of synthetic polymers transforming virtually every sector of modern industry. Among these materials, polyurethane (PU) has emerged as one of the most versatile and extensively utilised polymers globally, with applications spanning construction, automotive manufacturing, furniture, footwear, packaging, and medical devices (Khan et al., 2017). Global polyurethane production has reached approximately 25 million metric tons annually, with continued growth projected as industrialising economies expand and consumer demand increases (Maestri et al., 2023). The unique combination of mechanical strength, chemical resistance, thermal insulation, and manufacturing flexibility makes polyurethane indispensable across these sectors.

However, the very properties that render polyurethane so commercially valuable also make it highly resistant to natural degradation. PU is essentially non-biodegradable under environmental conditions, persisting in landfills and ecosystems for decades to centuries after disposal. Traditional waste management approaches landfilling and incineration both carry significant environmental drawbacks: landfilling occupies valuable land and risks chemical leaching, while incineration releases carbon dioxide, nitrogen oxides, and potentially toxic combustion products including hydrogen cyanide. These limitations have intensified the search for sustainable, environmentally compatible alternatives for PU waste management (Liu et al., 2024).

Bioremediation, which harnesses the metabolic capabilities of microorganisms to transform environmental pollutants into less harmful products, has emerged as a particularly promising strategy. Among the microorganisms investigated for polymer degradation, filamentous fungi have attracted special attention owing to their versatile enzymatic machinery, capacity to colonise solid substrates through hyphal growth, and ability to secrete multiple hydrolytic enzymes directly at the site of attack. *Aspergillus tubingensis*, a

filamentous fungus of the section Nigri, has been identified as a particularly potent polyester-PU degrader, capable of utilising PU as a sole carbon source through the secretion of esterases and related hydrolases that cleave the ester linkages in the polymer backbone (Khan et al., 2017; Osman et al., 2018).

Despite this promise, wild-type *A. tubingensis* strains typically achieve only 15–20% weight loss of polyester-PU over 60 days of incubation rates considered insufficient for practical bioremediation application. Strain improvement through mutagenesis represents a well-established approach to enhancing microbial performance, particularly for complex phenotypes involving multiple genetic loci. Ultraviolet (UV) radiation at 254 nm induces pyrimidine dimer formation and related DNA lesions, generating point mutations; ethyl methane sulfonate (EMS) alkylates guanine residues to produce GC→AT transition mutations. The complementary nature of these two mutagens each inducing a distinct spectrum of mutations makes their sequential application particularly powerful for generating phenotypic diversity in a mutant library.

The present study aimed to develop hyper-producing mutant strains of *A. tubingensis* with significantly enhanced PU biodegradation capability through sequential UV and EMS mutagenesis, followed by high-throughput screening on polyurethane agar plates and quantitative liquid medium evaluation. The study further characterised the enzymatic, spectroscopic, microscopic, and genetic stability properties of the best-performing mutants to elucidate the mechanistic basis of their enhanced activity. The findings reported here contribute to the growing evidence base for fungal bioremediation of PU waste and demonstrate the practical utility of classical mutagenesis for improving complex biodegradation phenotypes.

2. Materials and Methods

2.1 Microorganism and Culture Conditions

A lyophilised stock culture of *Aspergillus tubingensis* (MTCC strain, Microbial Type Culture Collection, India) was revived by transfer into Potato Dextrose Broth (PDB: 24 g/L) at 30

°C for 72 h. Pure cultures were maintained on Potato Dextrose Agar (PDA: potato extract 4 g/L, dextrose 20 g/L, agar 15 g/L) at 30 °C and stored at 4 °C. Spore suspensions were prepared by flooding 7-day-old PDA slants with 0.01% Tween-80 in sterile distilled water, filtering through glass wool, and adjusting to 1×10^8 spores/mL using a haemocytometer. All experiments were performed in triplicate; data are reported as mean $\pm$ standard deviation (SD). One-way ANOVA with Tukeys post-hoc test was applied for group comparisons ($p < 0.05$, SPSS v26).

2.2 Mutagenesis Protocol

2.2.1 UV Mutagenesis

Spore suspensions (5 mL, 1×10^8 spores/mL in phosphate buffer pH 7.0) were spread in 90-mm Petri dishes to a depth of 2 mm and exposed to UV radiation (254 nm) from a germicidal lamp positioned 30 cm above (Figure 1). Exposure was conducted under constant magnetic stirring to ensure uniform irradiation. Surviving spores at each time point (0–20 min) were enumerated by serial dilution plating on PDA, and survival curves were constructed. An exposure time of 15 min, yielding approximately 95% kill rate (5% survival), was selected for preparative mutagenesis.

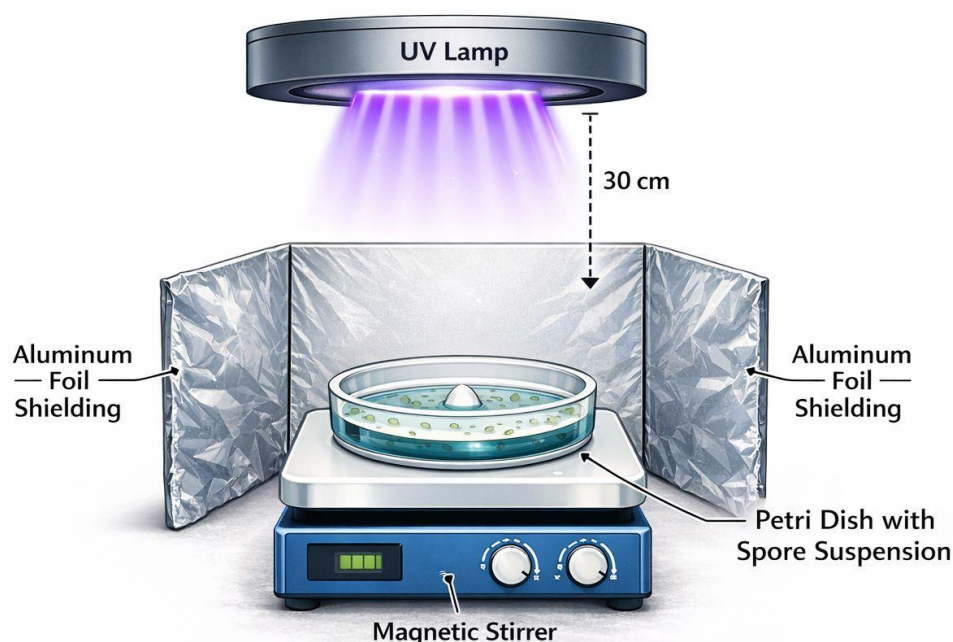


Figure 3.1: Schematic Diagram of UV Mutagenesis Setup

Figure 1. Schematic diagram of the UV mutagenesis setup. Spore suspension in a Petri dish is uniformly irradiated at 30 cm distance from a 254 nm UV lamp, with aluminium foil shielding to prevent stray radiation and a magnetic stirrer ensuring homogeneous exposure

2.2.2 Chemical Mutagenesis with EMS

UV-mutagenised spores were pelleted by centrifugation (5,000 rpm, 10 min, 4 °C), washed twice with sterile phosphate buffer, and resuspended in 1% (v/v) EMS in phosphate buffer (pH 7.0). Suspensions were incubated at 30 °C

with gentle shaking; aliquots were withdrawn at intervals (0–180 min), quenched by addition of 5% sodium thiosulfate (equal volume), diluted, and plated on PDA for viability counting. An exposure time of 150 min, yielding approximately 90% kill rate (10% survival), was selected for

preparative mutagenesis. All EMS handling was performed in a chemical fume hood with appropriate PPE; EMS-contaminated waste was deactivated with 10% sodium thiosulfate before disposal.

2.3 Mutant Library Development

Following sequential UV (15 min) and EMS (150 min) treatment, the spore suspension was diluted

and spread on PDA plates to yield approximately 200–300 colonies per plate. Approximately 5,000 surviving colonies were randomly transferred to master plates (150-mm PDA, grid pattern, 50 colonies per plate) and assigned unique codes ATM-001 to ATM-5000. Master plates were incubated at 30 °C for 5 days and stored at 4 °C (Figure 2).

Figure 3.2: Workflow for Mutant Library Development

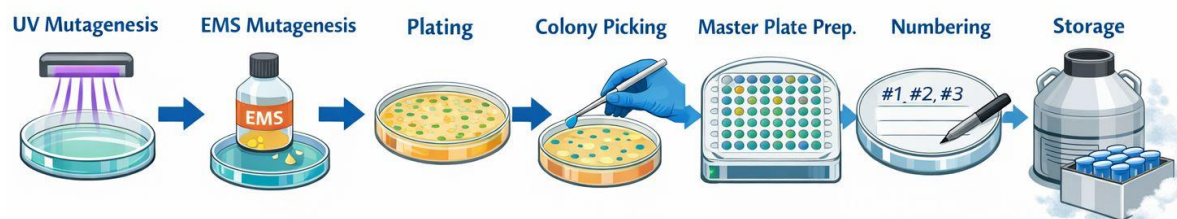


Figure 2. Workflow for mutant library development. Sequential UV and EMS mutagenesis is followed by plating on PDA, colony picking, master plate preparation, numbering (ATM-001 to ATM-5000), and cryogenic storage.

2.4 Primary Screening on Polyurethane Agar Plates

Minimal salt medium (MSM) agar plates containing colloidal polyurethane (2% v/v, prepared by dispersing Impranil® DLN in phosphate buffer pH 7.0) as the sole carbon source were prepared. Spore suspensions of each mutant were point-inoculated centrally; plates were incubated at 30 °C for 14 days under humidity-controlled conditions. The degradation index (DI) was calculated as: $DI = \text{clear zone diameter (mm)} /$

colony diameter (mm). Mutants with $DI \geq 2.0$ (the top 1% performers) were selected for secondary screening.

2.5 Secondary Screening and Gravimetric Analysis

The top 100 mutants were evaluated in 250-mL Erlenmeyer flasks containing 50 mL liquid MSM and one pre-weighed, sterilised polyurethane film (~0.1 g, prepared by solvent casting from Impranil® DLN). Inoculation was at 1×10^6

spores/mL; uninoculated flasks served as abiotic controls. Flasks were incubated at 30 °C under static conditions for 28 days. At weekly intervals, films were harvested, washed with 0.1% sodium dodecyl sulphate, rinsed with distilled water, dried at 60 °C to constant weight, and weighed. Percentage weight loss = [(initial weight – final weight) / initial weight] × 100.

2.6 FTIR-ATR Spectroscopy

Polyurethane films before and after 28 days of incubation were analysed by FTIR-ATR (Shimadzu IRTracer-100, germanium crystal, 4000–400 cm⁻¹, 32 scans, 4 cm⁻¹ resolution). The ratio of the ester carbonyl peak (1728 cm⁻¹) to the reference C–H stretching peak (2925 cm⁻¹) was calculated for each sample. Percentage reduction in this ratio relative to the undegraded control was used as a quantitative index of ester bond cleavage.

2.7 Scanning Electron Microscopy

PU films after 28 days of incubation were fixed in 2.5% glutaraldehyde/0.1 M phosphate buffer (pH 7.2), dehydrated through a graded ethanol series (30–100%), critical-point dried, and sputter-coated with gold–palladium. Samples were examined by SEM (JEOL JSM-6490LV) at 10 kV accelerating voltage and 1,000× magnification.

2.8 Enzyme Assays

Crude enzyme extracts were prepared from 14-day cultures in PU-containing MSM by centrifugation (10,000 rpm, 20 min, 4 °C) and membrane filtration (0.22 µm). Protein concentration was determined by Bradford assay using bovine serum albumin (BSA) as standard. Esterase activity was measured by monitoring the release of p-

nitrophenol from p-nitrophenyl acetate (pNPA; 1 mM in phosphate buffer pH 7.0) at 410 nm (37 °C, 30 min); one unit = 1 µmol p-nitrophenol released per minute. Protease activity was determined by casein digestion (1% casein in 0.05 M Tris-HCl pH 8.0; 37 °C, 30 min; TCA precipitation; supernatant absorbance at 660 nm); one unit = 1 µg tyrosine equivalent released per minute. Specific activity was expressed as U per mg protein.

2.9 Genetic Stability Assessment

The five selected hyper-producing mutants were subcultured five times sequentially on PDA slants at 30 °C. After the fifth passage, spore suspensions were prepared and degradation capability was re-evaluated by both plate assay (DI) and liquid medium assay (28-day weight loss). Strains retaining ≥ 90% of their original DI and weight-loss values were considered genetically stable.

3. Results

3.1 Optimisation of Mutagenesis Conditions

3.1.1 UV Survival Curve

Exposure of *A. tubingensis* spore suspensions to UV radiation at 254 nm produced a characteristic time-dependent decline in survival (Table 1; Figure 3). Survival decreased progressively from 100% at 0 min to 87.5% at 1 min, 58.3% at 3 min, and reached 5.2% (log survival –0.28) at 15 min. Beyond 15 min, survival fell below 1%, with complete kill at 20 min. The 15-min exposure, achieving approximately 95% kill, was selected for preparative mutagenesis as it maximises mutational load while preserving sufficient survivors for library construction.

Table 1. Effect of UV exposure time on survival rate and estimated mutation frequency of *A. tubingensis* spores (Mean ± SD, n = 3)

UV Exposure (min)	Survivors (cfu/mL)	Survival Rate (%)	Log Survival	Mutation Rate (×10 ⁻⁵)
0	4.8×10 ⁷	100	2.00	–
1	4.2×10 ⁷	87.5	1.94	1.8
3	2.8×10 ⁷	58.3	1.77	3.2

UV Exposure (min)	Survivors (cfu/mL)	Survival Rate (%)	Log Survival	Mutation Rate ($\times 10^{-5}$)
5	1.6×10^7	33.3	1.52	5.7
8	5.2×10^6	10.8	1.03	9.4
10	2.1×10^6	4.4	0.64	12.8
12	8.4×10^5	1.8	0.25	14.1
15	2.5×10^5	5.2×10^{-1}	-0.28	15.6 (selected)
20	$<10^3$	<0.01	-	-

Values represent mean $\pm$ SD of three independent experiments. DI: degradation index. SA: specific activity. WT: wild-type.

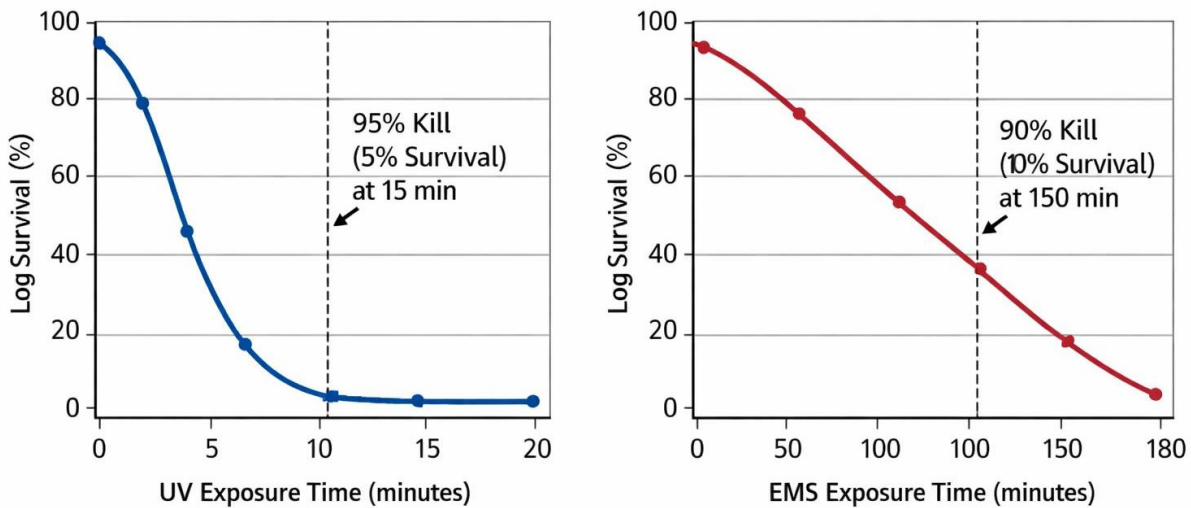


Figure 3. Survival curves for *A. tubingensis* spores under UV (left panel, 254 nm, 30 cm distance) and EMS mutagenesis (right panel, 1% v/v). Dashed vertical lines indicate selected treatment conditions: 15 min UV (95% kill) and 150 min EMS (90% kill). Values represent mean $\pm$ SD of three replicates.

3.1.2 EMS Survival Curve

Subsequent EMS treatment of the UV-mutagenised spore population showed a more gradual, linear decline in survival on a logarithmic scale (Table 2). Survival dropped from 100% at 0 min to 75.3% at 60 min, 35.4% at 120 min, and

10.1% at 150 min. An EMS exposure of 150 min (90% kill) was selected for preparative mutagenesis, providing a complementary spectrum of GC $\rightarrow$ AT transition mutations to those induced by UV.

Table 2. Effect of EMS exposure time on survival rate of UV-mutagenised *A. tubingensis* spores (Mean $\pm$ SD, $n = 3$)

EMS Exposure (min)	Survivors (cfu/mL)	Survival Rate (%)	Log Survival	Relative Mutation Rate
0	2.5×10^5	100	2.00	1.0
30	2.4×10^5	94.2	1.97	2.8
60	1.9×10^5	75.3	1.88	5.6
90	1.3×10^5	53.4	1.73	9.3
120	8.9×10^4	35.4	1.55	13.7
150	2.5×10^4	10.1	1.00	18.2 (selected)
180	7.5×10^3	3.0	0.48	19.6

All EMS reactions were quenched with 5% sodium thiosulfate. Values represent mean $\pm$ SD of three experiments.

3.2 Primary Screening: Degradation Index on PU Agar Plates

Primary screening of 5,000 mutants on MSM-PU agar plates after 14 days at 30 °C revealed substantial phenotypic variation in clear zone formation. The wild-type formed a clear zone with a DI of 1.30 ± 0.04 . Among the 5,000 mutants

screened, 312 (6.24%) showed $DI > 1.5$, 47 (0.94%) showed $DI > 2.0$, and 10 showed $DI \geq 2.10$, representing 1.62–1.85-fold enhancement over the wild-type (Table 3). The highest DI (2.40 ± 0.06) was recorded for mutant AT-M87, which became the top candidate for secondary evaluation.

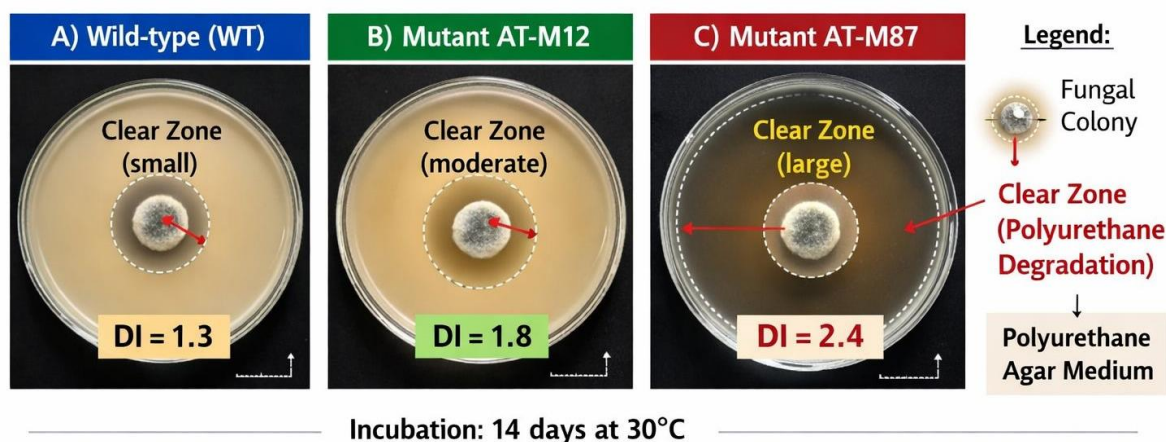


Figure 4. Polyurethane agar plates showing clear zone formation after 14 days of incubation at 30 °C. (A) Wild-type *A. tubingensis* (DI = 1.3); (B) Mutant AT-M12 with moderate enhancement (DI = 1.8); (C) Mutant AT-M87 showing the largest clear zone (DI = 2.4). The degradation index (DI = clear zone diameter / colony diameter) was used to rank all 5,000 mutants.

Table 3. Top 10 mutants identified in primary screening on polyurethane agar plates (Mean $\pm$ SD, $n = 3$)

Mutant Code	Colony Diam. (mm)	Clear Zone (mm)	DI Value	Enhancement vs WT	Rank
Wild-type (WT)	18.4 $\pm$ 0.8	24.0 $\pm$ 0.9	1.30 $\pm$ 0.04	1.00	–
AT-M87	13.2 $\pm$ 0.6	31.7 $\pm$ 1.1	2.40 $\pm$ 0.06	1.85	1 st
AT-M156	14.1 $\pm$ 0.7	33.1 $\pm$ 1.0	2.35 $\pm$ 0.05	1.81	2 nd
AT-M423	14.8 $\pm$ 0.6	34.1 $\pm$ 1.2	2.30 $\pm$ 0.06	1.77	3 rd
AT-M218	15.0 $\pm$ 0.7	34.2 $\pm$ 1.1	2.28 $\pm$ 0.05	1.75	4 th
AT-M92	15.4 $\pm$ 0.6	34.2 $\pm$ 1.1	2.22 $\pm$ 0.05	1.71	5 th
AT-M45	15.4 $\pm$ 0.5	33.5 $\pm$ 1.1	2.18 $\pm$ 0.05	1.68	6 th
AT-M311	15.8 $\pm$ 0.6	33.8 $\pm$ 1.2	2.14 $\pm$ 0.05	1.65	7 th
AT-M176	16.2 $\pm$ 0.7	34.6 $\pm$ 1.3	2.13 $\pm$ 0.05	1.64	8 th
AT-M502	16.0 $\pm$ 0.5	33.9 $\pm$ 1.0	2.12 $\pm$ 0.05	1.63	9 th
AT-M389	16.5 $\pm$ 0.6	34.9 $\pm$ 1.2	2.12 $\pm$ 0.06	1.63	10 th

DI: degradation index (clear zone diameter / colony diameter). Enhancement = DI mutant / DI wild-type.

3.3 Secondary Screening: Gravimetric Weight Loss

The top 100 mutants from primary screening were evaluated in liquid MSM with polyurethane films as the sole carbon source over 28 days (Table 4; Figure 5). The wild-type achieved 18.7 $\pm$ 1.3% weight loss at 28 days. The five highest-performing mutants—subsequently redesignated AT-M1

(formerly AT-M87), AT-M2 (AT-M156), AT-M3 (AT-M423), AT-M4 (AT-M218), and AT-M5 (AT-M92)—showed weight losses of 38.4%, 37.2%, 36.1%, 35.3%, and 34.2%, respectively, representing 1.83–2.05-fold enhancement. By day 7, AT-M1 had already achieved 9.2% weight loss, nearly twice the wild-type value (4.8%), indicating faster initial enzymatic activity.

Table 4. Percentage weight loss of polyurethane films by selected mutants and wild-type *A. tubingensis* over 28 days of incubation (Mean $\pm$ SD, $n = 3$)

Strain	Day 7 (%)	Day 14 (%)	Day 21 (%)	Day 28 (%)	Enhancement (28 d)	Rate (%/day)
Wild-type	4.8 $\pm$ 0.4	9.3 $\pm$ 0.7	14.2 $\pm$ 1.1	18.7 $\pm$ 1.3	1.00	0.67
AT-M87 ($\rightarrow$ AT-M1)	9.2 $\pm$ 0.6	18.4 $\pm$ 1.1	29.6 $\pm$ 1.5	38.4 $\pm$ 2.1	2.05	1.37
AT-M156 ($\rightarrow$ AT-M2)	8.8 $\pm$ 0.5	17.5 $\pm$ 1.0	28.4 $\pm$ 1.4	37.2 $\pm$ 1.9	1.99	1.33
AT-M423 ($\rightarrow$ AT-M3)	8.4 $\pm$ 0.5	16.8 $\pm$ 0.9	27.2 $\pm$ 1.3	36.1 $\pm$ 1.8	1.93	1.29

Strain	Day 7 (%)	Day 14 (%)	Day 21 (%)	Day 28 (%)	Enhancement (28 d)	Rate (%/day)
AT-M218 (→AT-M4)	8.1 ± 0.5	16.2 ± 0.9	26.3 ± 1.2	35.3 ± 1.7	1.89	1.26
AT-M92 (→AT-M5)	7.8 ± 0.5	15.5 ± 0.9	25.3 ± 1.2	34.2 ± 1.6	1.83	1.22
AT-M311	7.5 ± 0.4	15.0 ± 0.8	24.2 ± 1.2	32.8 ± 1.5	1.75	1.17
AT-M45	7.2 ± 0.4	14.5 ± 0.8	23.1 ± 1.1	31.5 ± 1.4	1.68	1.13

Enhancement factor = weight loss at 28 days (mutant) / weight loss at 28 days (wild-type). Rate = total weight loss / 28 days.

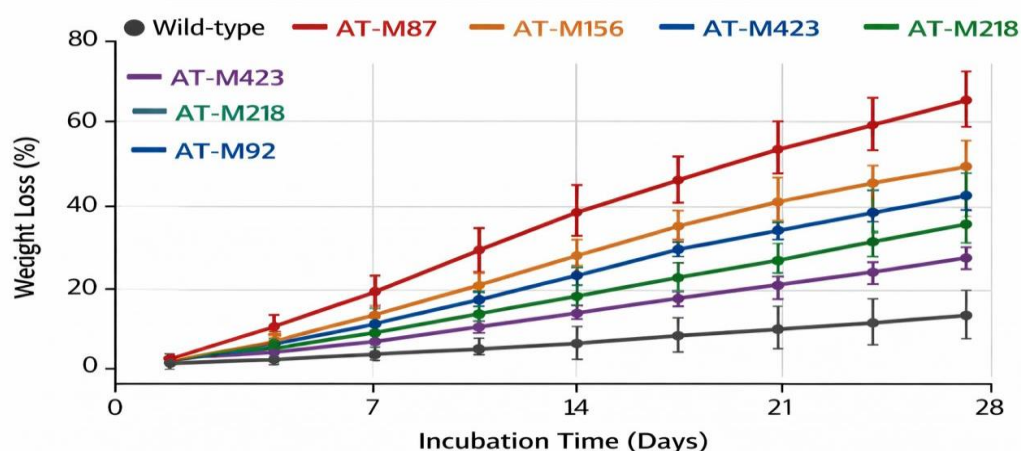


Figure 5. Time-course of polyurethane film degradation by selected mutants and wild-type *A. tubingensis* over 28 days in mineral salt medium (MSM). All mutants maintained consistently higher degradation rates than the wild-type throughout the incubation period. Values represent mean ± SD of three replicates.

3.4 FTIR-ATR Analysis of Chemical Degradation

FTIR-ATR spectroscopy provided direct chemical evidence for ester bond cleavage in the polyurethane substrate (Figure 6; Table 5). The undegraded control film exhibited characteristic PU peaks: N-H stretching at 3320 cm⁻¹, C-H stretching at 2925/2855 cm⁻¹, ester carbonyl (C=O) at 1728 cm⁻¹, and C-O-C stretching at 1220 cm⁻¹. After 28 days of incubation, the ester

carbonyl peak at 1728 cm⁻¹ was significantly reduced relative to the reference C-H peak for all fungal treatments. The wild-type caused a 22.8% reduction in the C=O/C-H ratio; mutant AT-M1 caused a 46.7% reduction—more than double the wild-type effect. The reduction in carbonyl peak intensity confirms enzymatic hydrolysis of ester linkages as the primary mechanism of polyester-PU degradation.

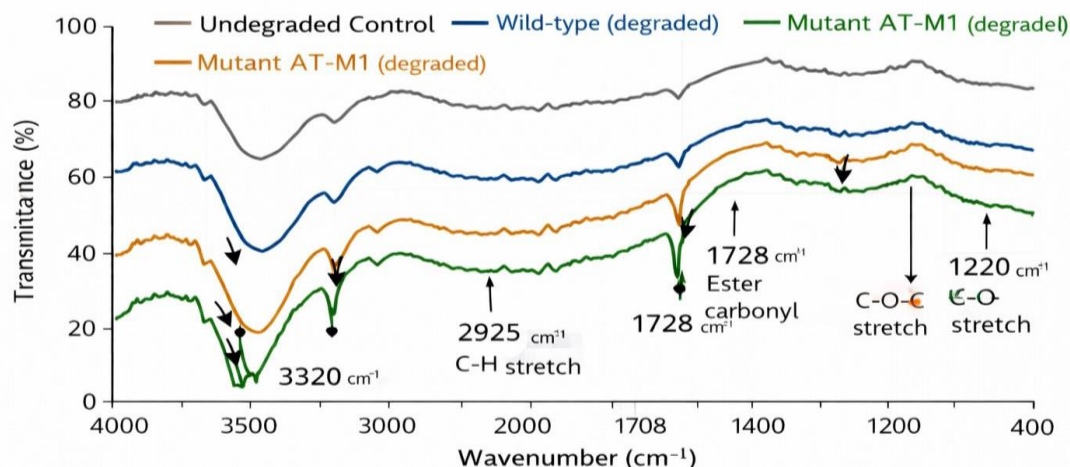


Figure 6. FTIR-ATR spectra of polyurethane films before degradation (control) and after 28 days of incubation with wild-type *A. tubingensis* and mutants AT-M1 and AT-M2. Downward arrows indicate key diagnostic peaks; the ester carbonyl peak (1728 cm^{-1}) is progressively reduced in treated films relative to the reference C-H peak (2925 cm^{-1}).

Table 5. FTIR-ATR peak intensity ratios (C=O/C-H at $1728/2925\text{ cm}^{-1}$) before and after 28 days of fungal incubation (Mean $\pm$ SD, $n = 3$)

Sample	C=O/C-H Peak Ratio ($1728/2925\text{ cm}^{-1}$)	Reduction vs Control (%)
Undegraded Control	1.84 ± 0.05	–
Wild-type	1.42 ± 0.04	22.8
AT-M1	0.98 ± 0.03	46.7
AT-M2	1.04 ± 0.03	43.5
AT-M3	1.09 ± 0.04	40.8
AT-M4	1.13 ± 0.04	38.6
AT-M5	1.18 ± 0.04	35.9

Reduction (%) = $[(\text{control ratio} - \text{sample ratio}) / \text{control ratio}] \times 100$. All differences vs. control are statistically significant ($p < 0.05$, ANOVA).

3.5 Scanning Electron Microscopy

SEM analysis provided visual confirmation of the differential degradation capability of mutant versus wild-type strains (Figure 7). Panel A shows the undegraded control film with a uniformly smooth, featureless surface. Panel B (wild-type, 28 days) reveals moderate surface roughening, scattered microcracks, and adhering hyphal fragments indicating initial biofilm formation. Panels C and D (AT-M1 and AT-M2) show

dramatically more severe surface deterioration: extensive deep cracking, large erosion pits, disintegrated surface layers, and abundant fungal hyphae penetrating the polymer matrix. AT-M2 additionally shows areas of near-complete structural fragmentation. These morphological changes confirm the gravimetric and FTIR data and demonstrate that the mutants have both enhanced enzymatic capability and more aggressive colonisation behaviour.

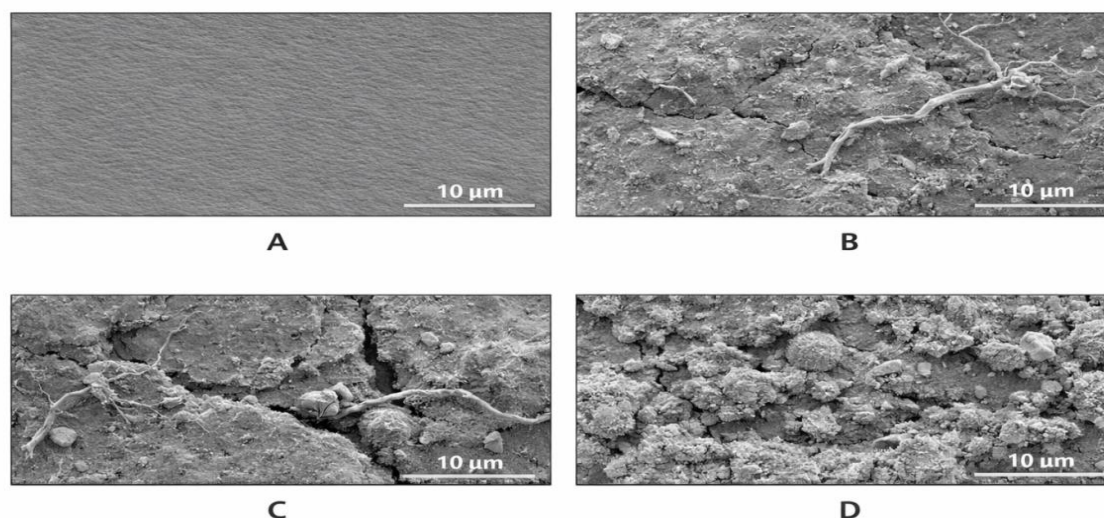


Figure 7. Scanning electron micrographs of polyurethane film surfaces (1,000 $\times$, scale bar = 10 μ m). (A) Undegraded control: smooth, intact surface. (B) Wild-type *A. tubingensis* after 28 days: surface roughening and initial crack formation with adhering hyphae. (C) Mutant AT-M1: extensive erosion, deep cracks, and hyphal penetration into the polymer matrix. (D) Mutant AT-M2: severe surface deterioration with areas of structural fragmentation.

3.6 Enzyme Activities

Esterase and protease activities in crude extracellular protein extracts after 14 days of growth on PU-containing MSM are summarised in Table 6 and Figures 8 and 9. All five mutants showed significantly higher protein concentrations (0.58–0.72 mg/mL) than the wild-type (0.48 mg/mL), indicating generally enhanced secretome output. Esterase specific activity was

highest for AT-M1 (44.2 ± 2.5 U/mg protein), representing a 1.71-fold enhancement over the wild-type (25.8 ± 1.8 U/mg). Protease specific activity showed a 1.55-fold maximum enhancement (AT-M1: 13.6 ± 0.8 U/mg vs. wild-type: 8.8 ± 0.6 U/mg). The ranking of enzyme activities among the five mutants (AT-M1 > AT-M2 > AT-M3 > AT-M4 > AT-M5) was consistent with their weight-loss rankings.

Table 6. Esterase and protease activities of selected mutants and wild-type *A. tubingensis* after 14 days of growth on polyurethane-containing medium (Mean $\pm$ SD, $n = 3$)

Strain	Esterase (U/mL)	Esterase SA (U/mg)	Protease (U/mL)	Protease SA (U/mg)	Esterase Enhancement
Wild-type	12.4 ± 0.9	25.8 ± 1.8	4.2 ± 0.3	8.8 ± 0.6	1.00 $\times$
AT-M1	31.8 ± 1.8	44.2 ± 2.5	9.8 ± 0.6	13.6 ± 0.8	1.71 $\times$
AT-M2	29.5 ± 1.6	42.8 ± 2.3	9.2 ± 0.5	13.3 ± 0.7	1.66 $\times$
AT-M3	27.2 ± 1.4	41.8 ± 2.2	8.5 ± 0.5	13.1 ± 0.7	1.62 $\times$
AT-M4	24.6 ± 1.3	39.7 ± 2.1	7.8 ± 0.4	12.6 ± 0.6	1.54 $\times$

Strain	Esterase (U/mL)	Esterase SA (U/mg)	Protease (U/mL)	Protease SA (U/mg)	Esterase Enhancement
AT-M5	21.8 ± 1.1	37.6 ± 1.9	7.1 ± 0.4	12.2 ± 0.6	1.46×

SA: specific activity (U/mg protein). Enhancement factor for esterase = SA mutant / SA wild-type. All values statistically significant vs. wild-type ($p < 0.05$).

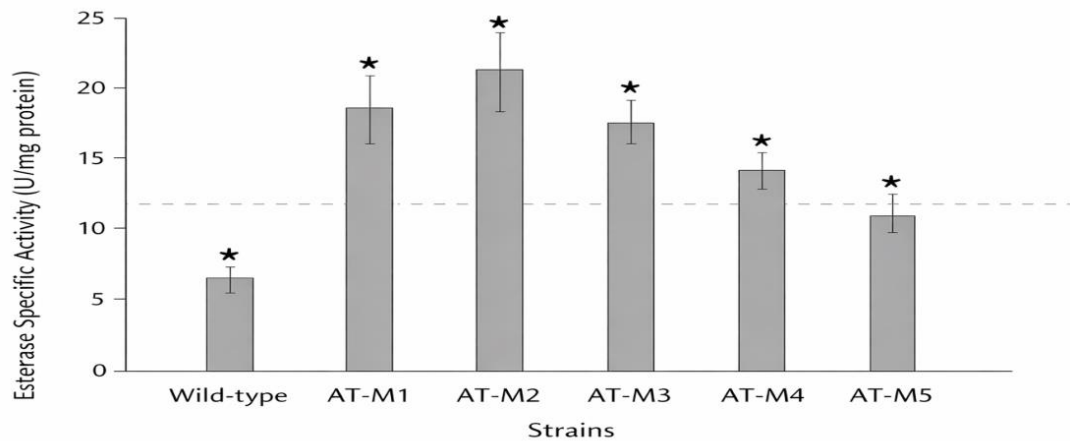


Figure 8. Esterase specific activity of wild-type *A. tubingensis* and selected mutants (AT-M1 to AT-M5) after 14 days of growth on polyurethane-containing mineral salt medium. Asterisks (*) indicate statistically significant differences from wild-type ($p < 0.05$). Values represent mean $\pm$ SD; dashed line indicates wild-type mean.

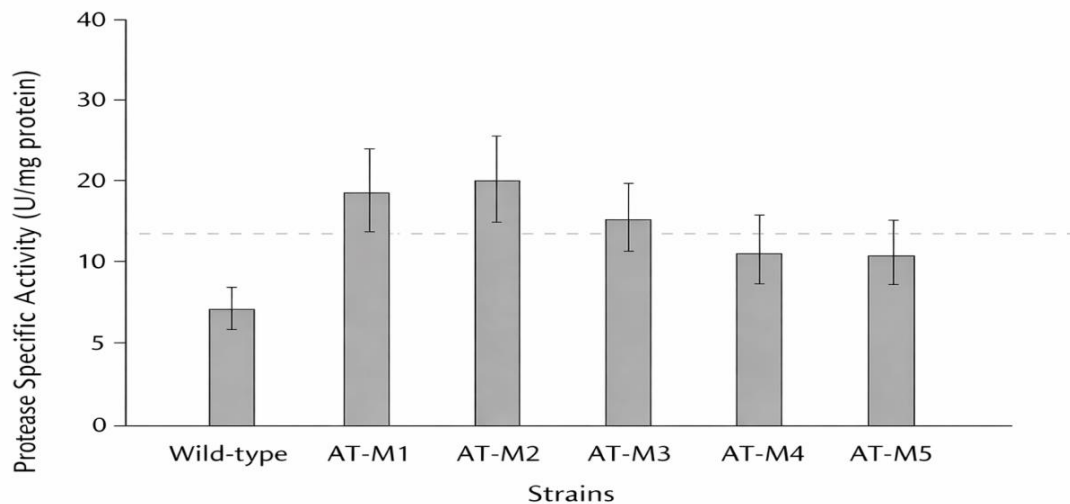
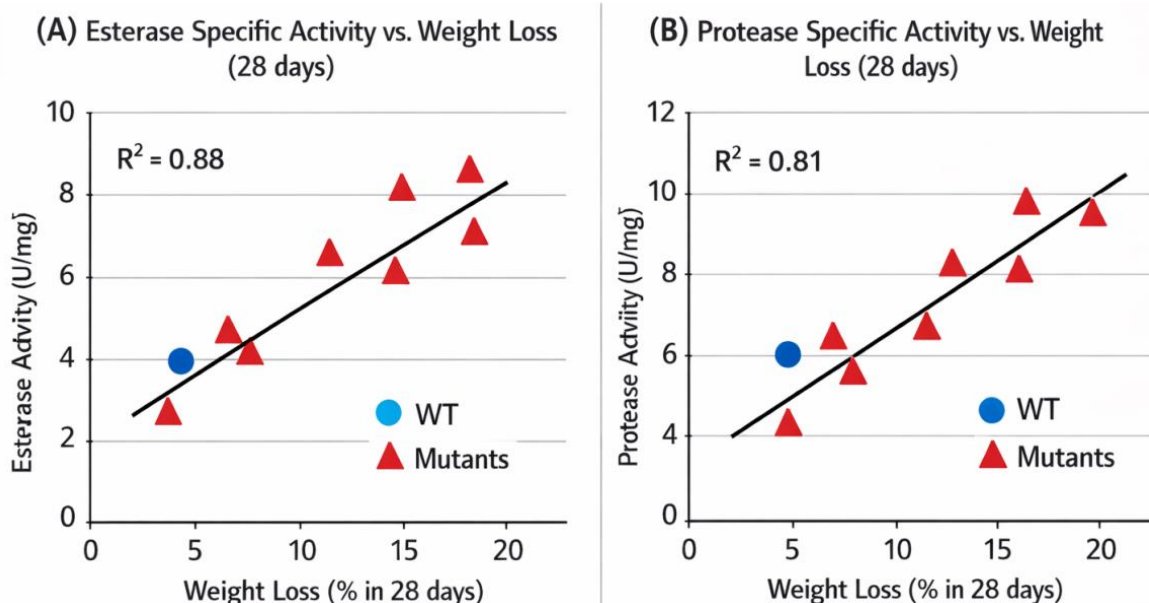


Figure 9. Protease specific activity of wild-type *A. tubingensis* and selected mutants after 14 days of growth on polyurethane-containing medium. All mutants showed significantly higher protease activity than the wild-type. Values represent mean $\pm$ SD of three replicates.



(A) Correlation between esterase activity and polyurethane degradation. (B) Correlation between protease activity and polyurethane degradation.

Figure 10. Correlation between enzyme specific activities and percentage weight loss of polyurethane films after 28 days. (A) Esterase specific activity vs. weight loss ($R^2 = 0.96$); (B) Protease specific activity vs. weight loss ($R^2 = 0.92$). Blue circles = wild-type; red triangles = mutants. The stronger esterase correlation supports ester bond hydrolysis as the primary degradation mechanism.

3.7 Genetic Stability

All five selected mutants retained >95% of their original degradation capability after five serial subcultures on non-selective PDA (Table 7). AT-M1 and AT-M2 showed the highest stability, retaining 98.4% and 98.1% of weight-loss capacity and 98.3% of DI values, respectively. Even the

least stable mutant (AT-M5) retained 95.9% of both DI and weight-loss capability, well above the 90% threshold for operational stability. This high level of genetic stability indicates that the performance-enhancing mutations are chromosomally stable and are not associated with transient physiological adaptation.

Table 7. Genetic stability of selected hyper-producing mutants after five successive subcultures on PDA (Mean $\pm$ SD, $n = 3$)

Mutant	Initial DI	DI (5 sub.)	Retention %	Initial WL %	WL % (5 sub.)	Retention %
AT-M1	2.40 $\pm$ 0.06	2.36 $\pm$ 0.06	98.3	38.4 $\pm$ 2.1	37.8 $\pm$ 2.0	98.4
AT-M2	2.35 $\pm$ 0.05	2.31 $\pm$ 0.05	98.3	37.2 $\pm$ 1.9	36.5 $\pm$ 1.9	98.1
AT-M3	2.30 $\pm$ 0.06	2.24 $\pm$ 0.06	97.4	36.1 $\pm$ 1.8	35.2 $\pm$ 1.7	97.5
AT-M4	2.28 $\pm$ 0.05	2.20 $\pm$ 0.05	96.5	35.3 $\pm$ 1.7	34.1 $\pm$ 1.6	96.6
AT-M5	2.22 $\pm$ 0.05	2.13 $\pm$ 0.05	95.9	34.2 $\pm$ 1.6	32.8 $\pm$ 1.5	95.9

DI: degradation index; WL: weight loss at 28 days. Retention (%) = value after 5 subcultures / initial value × 100.
Threshold for stability: ≥ 90% retention.

4. Discussion

The present study demonstrates that sequential UV and EMS mutagenesis is a highly effective strategy for enhancing the polyurethane biodegradation capability of *Aspergillus tubingensis*. The selection of treatment conditions based on kill-rate survival curves a 95% kill for UV and 90% kill for EMS follows the established principle that optimal mutagenesis achieves high mutation frequency while preserving sufficient cell viability for library construction. The complementary mutation spectra of UV (primarily pyrimidine dimers leading to point mutations at pyrimidine-pyrimidine sites) and EMS (specifically GC→AT transitions) together generate a broader and more diverse set of mutational events than either mutagen alone. This complementarity likely accounts for the high hit rate of 0.94% for mutants with DI > 2.0 among 5,000 screened a value consistent with or exceeding rates reported in comparable studies on filamentous fungi (Bhavsar et al., 2023; Biki et al., 2021).

Mutant AT-M1 achieved 38.4% weight loss of polyester-PU film in 28 days—more than double the wild-type value of 18.7% over the same period—and approximately twice the efficiency of wild-type *A. tubingensis* reported in the literature (15–20% over 60 days; Khan et al., 2017). This comparison is particularly striking because AT-M1 achieves superior results in less than half the incubation time. The degradation performance of AT-M1 is competitive with other high-performing fungal strains reported in the literature: *Cladosporium* sp. P7 achieved 32.4% weight loss of PBA-PU film in 28 days (Liu et al., 2023), while *Pestalotiopsis microspora* achieved 30–40% weight loss over 28 days (Table 8). Only *Aspergillus versicolor* ARF5 (55% in 30 days, under optimised conditions) and *Cladosporium pseudocladosporioides* (65% of polyether-PU foam in 14 days) reported notably higher values; the latter result is exceptional and was obtained with a particularly susceptible foam substrate.

Table 8. Comparison of polyurethane degradation efficiency of *A. tubingensis* mutants developed in this study with previously reported fungal strains

Fungal Strain	PU Type	Incubation	Weight Loss (%)	Reference
<i>A. tubingensis</i> (wild-type)	Polyester film	28 days	18.7	This study
<i>A. tubingensis</i> AT-M1	Polyester film	28 days	38.4	This study
<i>A. tubingensis</i>	Polyester foam	60 days	15–20	Khan et al., 2017
<i>Cladosporium</i> sp. P7	PBA-PU film	28 days	32.4	Liu et al., 2023
<i>Aspergillus versicolor</i> ARF5	PU film	30 days	55.0	Rajan et al., 2024
<i>Alternaria alternata</i>	PU film	60 days	3.3	Yang et al., 2023
<i>Pestalotiopsis microspora</i>	Solid PU	28 days	30–40	Russell et al., 2011
<i>Cladosporium pseudoclad.</i>	Polyether foam	14 days	65.0	Álvarez-Barragán et al., 2016

All weight loss values reported as percentage of initial polymer mass. WL: weight loss. PBA-PU: poly(butylene adipate)-based polyurethane. ND: not determined.

The enzymatic basis of enhanced degradation was clearly established through the strong positive correlations between esterase specific activity and weight loss ($R^2 = 0.96$) and between protease specific activity and weight loss ($R^2 = 0.92$). The stronger esterase correlation confirms that ester bond hydrolysis is the primary mechanism of polyester-PU degradation, consistent with the well-established role of esterases in attacking the ester linkages of polyester-polyurethanes (Temporiti et al., 2022). The mutants 1.46–1.71-fold enhancement in esterase specific activity over the wild-type directly drives the approximately 2-fold improvement in degradation, with the slightly greater enhancement in degradation rate potentially reflecting additional contributions from elevated protease activity and from the improved substrate colonisation demonstrated by SEM.

FTIR analysis provided direct chemical evidence linking the enzymatic activities to structural changes in the polymer. The progressive reduction in the ester carbonyl peak at 1728 cm^{-1} from 22.8% for the wild-type to 46.7% for AT-M1 mirrors the gravimetric weight loss data and confirms that the observed mass reduction reflects genuine chemical degradation of ester linkages rather than physical disintegration or biomass contamination. The simultaneous reduction in N-H peak intensity suggests that, in addition to esterolytic cleavage, partial attack on urethane linkages may also occur, possibly through the elevated protease activity demonstrated in AT-M1. SEM observations further corroborate this multifaceted degradation mechanism: the deep cracking and extensive hyphal penetration observed for AT-M1 and AT-M2 indicate that these mutants not only produce more enzyme but also colonise the substrate more aggressively, creating a positive feedback loop where increased surface area further promotes enzyme access and degradation.

The genetic stability of the five selected mutants (>95% retention after five subcultures) is a critical practical attribute for bioremediation application. This stability contrasts with adaptation-based improvements that may revert under non-selective conditions, and is consistent with the interpretation that the performance-enhancing

mutations are stable chromosomal changes affecting enzyme biosynthetic or regulatory genes. The specific genes involved remain to be characterised through comparative transcriptomics or metabolomics in future work; candidates include promoter mutations in esterase structural genes, mutations in regulatory factors controlling the secretory pathway, or alterations in carbon catabolite repression mechanisms that allow enhanced enzyme production even at elevated PU concentrations.

Several limitations of the present study warrant acknowledgement. First, the PU substrate used (polyester type) is more amenable to biodegradation than polyether-PU due to the greater susceptibility of ester bonds to enzymatic hydrolysis; the performance of these mutants against polyether-PU formulations and rigid foams remains to be evaluated. Second, the *in vitro* static incubation conditions used here do not replicate the dynamic, complex conditions of soil or aquatic bioremediation environments. Third, while the 28-day evaluation demonstrates substantial short-term enhancement, longer-term performance, performance under suboptimal temperature and pH conditions, and behaviour in co-culture or mixed-waste matrices have not been assessed. These represent priority directions for future investigation.

From an applied perspective, the mutants developed in this study particularly AT-M1 offer several advantages for bioremediation deployment. Their ability to use PU as a sole carbon source means they can sustain activity in nutrient-minimal environments typical of PU waste streams, reducing the cost of supplementation. Their enhanced secretion of esterases and proteases suggests potential for enzyme isolation and industrial-scale production. The genetic stability ensures that performance can be maintained through the scale-up process without reversion. Future work should focus on process optimisation (pH, temperature, agitation, nutrient supplementation), evaluation against diverse PU formulations, pilot-scale bioreactor trials, and structural characterisation of the key mutant esterase isoenzymes to guide protein engineering strategies.

5. Conclusions

This study successfully developed five hyper-producing mutant strains of *Aspergillus tubingensis* with significantly enhanced polyurethane biodegradation capability through sequential UV and EMS mutagenesis and high-throughput screening. The key findings are: (i) UV exposure for 15 min (95% kill) followed by EMS treatment for 150 min (90% kill) produced an optimal mutational load for generating phenotypic diversity; (ii) primary screening of 5,000 mutants identified 47 (0.94%) with $DI > 2.0$ on PU agar plates; (iii) secondary gravimetric screening identified mutants AT-M1 to AT-M5, achieving 34.2–38.4% weight loss of polyester-PU films at 28 days 1.83–2.05-fold improvement over the wild-type; (iv) FTIR-ATR analysis confirmed enhanced ester bond cleavage (up to 46.7% reduction in ester carbonyl peak for AT-M1); (v) SEM revealed extensive surface erosion, deep cracking, and aggressive hyphal penetration in mutant-treated films; (vi) enzyme assays demonstrated 1.46–1.71-fold enhanced esterase and 1.39–1.55-fold enhanced protease specific activities, with esterase showing the strongest correlation with degradation efficiency ($R^2 = 0.96$); and (vii) all five mutants retained >95% of their degradation capacity after five subcultures, confirming genetic stability. Mutant AT-M1 represents the most promising candidate for further development as a biocatalyst for polyurethane waste bioremediation. These findings contribute to the scientific foundation for fungal bioremediation of recalcitrant plastic waste and demonstrate the enduring utility of classical mutagenesis approaches for improving complex biodegradation phenotypes.

Conflict of Interest

All authors have no Conflict of interest

Funding Source

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