Strain Improvement of *Bacillus Subtilis* for Enhanced α-Amylase Production Using UV and Ethidium Bromide Mutagenesis

Surbhi Rathor[1], Dr.Vinita Dheeran[2*], Dr. Harjinder Singh[3]

[1,3]Department of Botany Meerut College, Meerut, India

[2]Department of Botany M M H College, Ghaziabad, India

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ABSTRACT

Microbial α-amylase is an industrially important starch-hydrolyzing enzyme with applications spanning food processing, starch conversion, textiles, detergents, paper manufacture, fermentation and other biotechnological processes. The present study evaluated strain improvement of an amylolytic bacterial isolate, designated PSSR201801 and identified in the supplied study records as *Bacillus subtilis,* using physical and chemical mutagenesis. A rhizospheric soil sample collected near Meerut College, Meerut was processed by serial dilution and spread plating. Amylolytic colonies were screened on starch-containing minimal agar and visualized by iodine flooding, after which the selected isolate was purified by quadrant streaking. UV irradiation and ethidium bromide (EtBr) treatment were subsequently used to generate mutant populations, and colonies were re-screened for starch hydrolysis. The experimental record indicates that UV exposure progressively reduced recoverable colony numbers from approximately 220 at 5 min to 45 at 20 min, while EtBr treatment yielded 76 colonies at 6 µL and 32 colonies at 8 µL, with lower treatments reported as lawn growth. The 20-min UV-derived culture and a positive EtBr-derived culture were selected for subsequent enzyme production studies. Growth-curve measurements showed that PSSR201801 increased from an OD620 of 0 at inoculation to 0.33 at 48 h before declining to 0.25 at 72 h. Amylase production was assessed using the dinitrosalicylic acid (DNS) reducing-sugar assay, followed by partial purification through ammonium sulfate precipitation and dialysis. The supplied records report enhanced amylase activity following mutagenic treatment, including a stated doubling of enzyme activity after 20 min of UV exposure; however, replicate-level numerical enzyme-activity values were not available in the source files and therefore are not fabricated here. Overall, the study supports random mutagenesis as a practical route for generating candidate high-producing Bacillus variants and provides a foundation for subsequent molecular characterization, statistical optimization and scale-up.

Keywords: α-Amylase; Bacillus subtilis; strain improvement; UV mutagenesis; ethidium bromide; starch hydrolysis; DNS assay; submerged fermentation; partial purification; industrial biotechnology.

Research significance

The work integrates environmental isolation, plate-based screening, mutagenesis, fermentation, colorimetric enzyme estimation and downstream processing into a single strain-development workflow. Its principal value lies in demonstrating how a locally isolated Bacillus culture can be advanced from environmental screening toward an industrial enzyme-production candidate.

INTRODUCTION

Enzymes are central to modern industrial biotechnology because they catalyze reactions with high specificity under comparatively mild conditions. Among hydrolytic enzymes, amylases occupy a particularly important position because starch is an abundant, inexpensive and renewable polysaccharide feedstock. α-Amylase catalyzes endo-hydrolysis of α-1,4-glycosidic linkages in starch and related substrates, producing shorter dextrins and oligosaccharides. Microbial amylases have become especially attractive because microorganisms can be cultivated on inexpensive substrates, can secrete extracellular enzymes and can be manipulated through process optimization or strain improvement. Reviews of microbial amylases have emphasized their importance

in starch saccharification and applications in food, textile, brewing, detergent and other industries (Pandey et al., 2000; Sivaramakrishnan et al., 2006).

The industrial relevance of amylase is closely connected to the properties of the producing organism and enzyme. A useful production strain should grow reproducibly, secrete high levels of enzyme, tolerate the physicochemical conditions of fermentation and retain productivity over repeated cultivation. Bacillus species are widely studied for extracellular enzyme production because of their capacity for secretion and their physiological versatility. The supplied study identified the selected isolate as B. subtilis using morphological and taxonomic characteristics referenced to Bergey's Manual. The experimental objective was therefore not simply to isolate an amylase producer, but to improve its productive phenotype by generating and selecting mutants.

Classical strain improvement relies on creating genetic diversity followed by selection. Physical mutagens such as ultraviolet radiation can produce DNA lesions, including pyrimidine dimers, whereas chemical mutagens can alter DNA bases or interfere with replication. Random mutagenesis does not require prior knowledge of the genetic determinants controlling enzyme production and can therefore be useful when the phenotype is complex. Previous studies have demonstrated that mutagenesis can yield Bacillus variants with increased amylase productivity, although the magnitude and stability of improvement are strain- and process-dependent. For example, a stable high-yielding B. subtilis mutant with substantially greater α-amylase secretion was reported following mutagenic treatment (Shah et al., 1989), while more recent work has continued to use UV-based approaches for improved enzyme production and desirable enzyme properties.

The present study is therefore framed as a strain-improvement investigation in which environmental isolation, primary/secondary screening, UV and EtBr mutagenesis, fermentation, enzyme assay and partial purification are considered as linked stages of a development pipeline. Importantly, the supplied records contain qualitative evidence of improved production and several quantitative colony-count and growth measurements, but they do not provide a complete replicate dataset for enzyme activity. A scientifically defensible manuscript must distinguish measured values from qualitative observations and avoid assigning unsupported statistical significance.

Industrial relevance of α-amylase

α-Amylase is used in starch processing to reduce viscosity and generate fermentable or syrup-producing carbohydrates. In baking, controlled starch hydrolysis can influence dough handling, fermentation and bread quality. In textile processing, amylases are used in desizing, where starch-based sizing materials applied to yarn are removed before subsequent wet processing. In detergents, amylases assist in the degradation of starch-containing food residues. The enzyme is also relevant to pulp and paper processing, fermentation and biofuel production because starch must frequently be converted into soluble sugars before downstream microbial conversion. These applications make productivity, stability and cost important determinants of commercial value.

The supplied background document notes that microbial amylases have largely displaced chemical starch hydrolysis in industrial contexts because biological catalysts can provide controlled reactions under comparatively mild conditions. This transition illustrates a broader principle in industrial microbiology: improving the biological catalyst can reduce the burden on downstream process conditions. Consequently, strain improvement is not merely an academic exercise; it can influence volumetric productivity, fermentation duration, raw-material use and downstream processing requirements.

Rationale for strain improvement

Natural microbial isolates frequently produce enzymes at levels that are insufficient for economical industrial manufacture. Their productivity may be constrained by transcriptional regulation, secretion capacity, metabolic flux, enzyme stability or interactions between the organism and its culture environment. Random mutagenesis offers a route to explore phenotypic space without requiring an established genetic engineering platform.

Mutants are generated and then subjected to a selection or screening regime that enriches for the desired phenotype.

In the present work, starch hydrolysis on agar was used as the first phenotype-level indicator. A clear hydrolysis zone after iodine treatment provides a rapid visual indication that starch surrounding a colony has been degraded. This method is particularly useful for screening large numbers of colonies, while a quantitative reducing-sugar assay is more appropriate for comparing enzyme production in liquid culture. The study therefore used plate screening as an initial filter and DNS-based assay as a quantitative production assessment.

Research objectives

Objective	Experimental focus
Isolation	Recovery of culturable bacteria from rhizospheric soil near Meerut College.
Screening	Identification of starch-hydrolyzing colonies using minimal agar containing starch and iodine visualization.
Strain improvement	Generation of mutant populations using UV irradiation and EtBr treatment.
Selection	Recovery and re-screening of candidate mutants showing starch hydrolysis.
Production	Comparison of parent and selected mutant cultures under submerged fermentation conditions.
Estimation	Determination of reducing-sugar release using DNS assay at 540 nm.
Downstream processing	Partial purification through ammonium sulfate precipitation and dialysis.

MATERIALS AND METHODS

Experimental design

The experimental workflow consisted of six major stages: (i) collection of rhizospheric soil, (ii) isolation and purification of bacterial cultures, (iii) screening for amylolytic activity, (iv) mutagenesis and selection of candidate mutants, (v) submerged production of amylase, and (vi) partial purification of the crude enzyme. The workflow was reconstructed directly from the supplied materials-and-methods record. Where the source document uses the designation PSSR201801, that designation is retained.

Sample collection and isolation

Rhizospheric soil was collected near Meerut College, Meerut. The supplied protocol describes preparation of sterile saline and serial dilution of the soil suspension, followed by spread plating on nutrient agar. Plates were incubated at 37 °C for 24 h. Mixed cultures were examined according to colony shape, margin, elevation, texture, opacity, pigmentation and surface characteristics. A representative colony was selected and purified by discontinuous quadrant streaking on nutrient agar.

Figure 1. Rhizospheric soil sample collected near Meerut College, Meerut. Image reproduced from the supplied experimental record.

Colony morphology and purification

The selected colony associated with PSSR201801 was described as circular, with an entire margin, flat elevation, off-white pigmentation, smooth texture, hard surface and opaque appearance. Colony morphology was used as a practical preliminary criterion for differentiation before enzymatic screening. Pure culture establishment was performed by repeated streaking and incubation, producing a discrete culture suitable for subsequent assays.

Figure 2. Purified culture plate of PSSR201801 following streaking. Image reproduced from the supplied experimental record.

Screening for amylase production

Primary and secondary screening were performed on minimal agar medium supplemented with starch. The supplied protocol describes visual evaluation of growth followed by iodine flooding. Starch hydrolysis produces a clear region around an amylolytic colony because hydrolyzed starch does not retain the iodine-starch coloration observed in intact substrate. The isolate showing the desired hydrolysis phenotype was retained for further work.

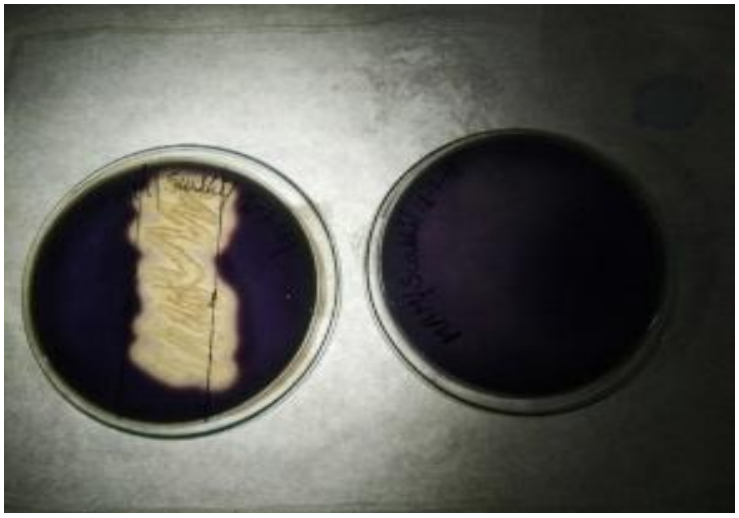

Figure 3. Starch-hydrolysis screening of the selected culture after iodine treatment; the clear zone represents substrate hydrolysis. Image reproduced from the supplied experimental record.

This plate assay is a qualitative or semi-quantitative screening method. It is particularly valuable for mutant selection because many colonies can be inspected comparatively, but the diameter of a clearing zone should not be treated as a direct measurement of volumetric enzyme activity without appropriate normalization. The subsequent liquid-culture DNS assay was therefore essential for production assessment.

UV mutagenesis

For physical mutagenesis, the supplied record describes exposure of PSSR201801 cultures to UV radiation for 5, 10, 15 and 20 min, with an untreated control. Following incubation, colonies were counted, candidate colonies were marked and purified by streaking, and the resulting cultures were re-screened for starch hydrolysis. A positive candidate was then grown in nutrient broth and assayed by the DNS method.

UV exposure	Recoverable colonies
Control	Lawn
5 min	≈220
10 min	150
15 min	85
20 min	45

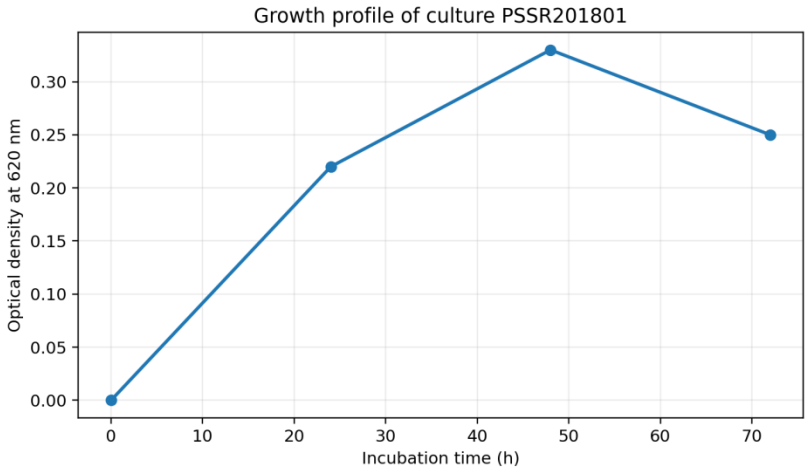

Figure 4. Growth curve of PSSR201801 derived from the supplied OD620 measurements (0–72 h).

Chemical mutagenesis with EtBr

Chemical mutagenesis was carried out using ethidium bromide (EtBr) treatment as described in the supplied experimental record. Treatments included 2, 4, 6 and 8 µL, alongside a control. Following incubation, treated cultures were spread on nutrient agar, colonies were recovered and candidate colonies were purified and screened for starch hydrolysis. The positive candidate was subsequently grown in nutrient broth for enzyme assay.

EtBr treatment	Reported colonies
Control	Lawn
2 µL	Lawn
4 µL	Lawn
6 µL	76
8 µL	32

The reduction in recoverable colonies with increasing treatment intensity is consistent with a general increase in biological stress, although the present data alone do not establish a quantitative dose-response relationship because the lower treatments were reported as lawn growth rather than enumerated colony counts. EtBr is a DNA-intercalating mutagen and is hazardous; therefore, all experimental handling must be performed under institutional biosafety and chemical-safety procedures. The present manuscript reports the supplied experimental history and does not propose new exposure conditions.

Figure 5. Representative culture plates associated with EtBr-mutagenesis experiments. Image reproduced from the supplied experimental record.

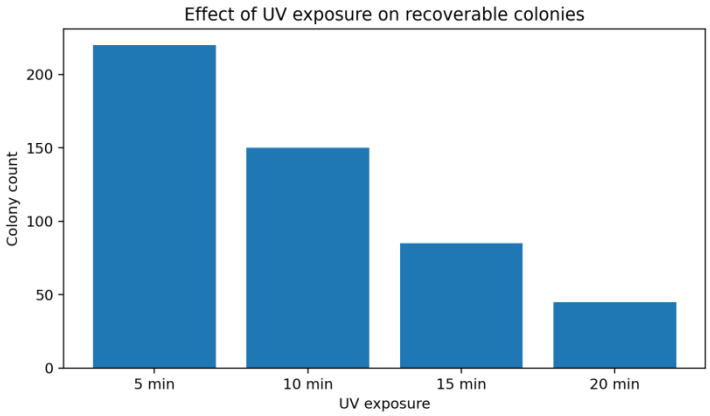

Figure 6. Effect of UV exposure duration on recoverable colony counts in the supplied dataset.

Enzyme production and quantitative assay

For production studies, the supplied record describes submerged fermentation in optimized production medium. The optimized formulation reported in the study contained beef extract, Na_2HPO_4, starch, sucrose, K_2HPO_4 and NaCl, with the working pH recorded as 4.0. Separate cultures representing the parent, chemically mutated and UV-mutated strains were inoculated into production medium and incubated on a shaker for 48 h. Crude enzyme was recovered from culture supernatant following centrifugation.

DNS assay

Amylase production was estimated through the dinitrosalicylic acid method for reducing sugars. Maltose standards were prepared, reacted with DNS reagent and heated, followed by dilution and measurement at 540 nm. A standard curve relating maltose concentration to absorbance was used to estimate reducing sugar release. The DNS approach is a classical colorimetric method for reducing sugars and remains widely used in enzyme-production studies (Miller, 1959). In the supplied study, the assay was used to compare production cultures after mutagenic treatment.

Figure 7. Culture plates showing bacterial growth associated with the isolation/screening workflow. Image reproduced from the supplied experimental record.

Partial purification

The crude enzyme-containing supernatant was subjected to ammonium sulfate precipitation under cold conditions. The precipitated protein was collected and dissolved in Tris buffer before dialysis against 100 mM Tris for 24 h. The dialyzed preparation was subsequently evaluated for enzyme activity by the DNS method. This represents partial purification rather than a complete purification scheme because no chromatographic separation, homogeneity assessment or specific-activity calculation was reported in the supplied files.

RESULTS

Isolation and morphological characterization

The study successfully recovered culturable bacteria from the collected rhizospheric soil and differentiated mixed cultures according to colony morphology. The selected PSSR201801 colony was described as circular, entire-margined, flat, off-white, smooth, hard and opaque. The culture was purified by quadrant streaking before enzyme screening. The supplied images document both mixed-culture growth and the subsequent purified culture.

Figure 8. Mixed bacterial growth observed during isolation from the soil sample. Image reproduced from the supplied experimental record.

Figure 9. Additional plate image from the isolation/screening stage. Image reproduced from the supplied experimental record.

Amylolytic screening

The selected isolate produced a visible starch-hydrolysis zone following iodine treatment, supporting its classification as an amylolytic candidate. The study then carried the culture forward into mutagenesis and quantitative enzyme-production experiments. The use of both plate screening and liquid assay is methodologically appropriate because the former provides high-throughput selection whereas the latter provides a numerical production endpoint.

Effect of UV treatment

UV exposure produced a clear decline in recoverable colony numbers with increasing treatment duration: approximately 220 colonies at 5 min, 150 at 10 min, 85 at 15 min and 45 at 20 min. The source record reports that the 20-min exposure was used to generate a selected UV-mutant culture and that the selected mutant displayed positive starch hydrolysis. The supplied abstract further states that 20 min of UV exposure doubled enzyme activity relative to the corresponding baseline. Because no raw activity values, replicate counts or statistical dispersion were supplied, the result is reported here as a source-record claim rather than independently re-calculated.

Effect of EtBr treatment

EtBr treatment also altered recoverable growth. The source record reports lawn growth at 2 and 4 µL and enumerated colonies of 76 and 32 at 6 and 8 µL, respectively. Two colonies were selected from the treated plates for purification, after which one candidate showed a positive hydrolysis phenotype. These observations indicate that mutagenic treatment generated a recoverable population from which phenotypic selection could be performed.

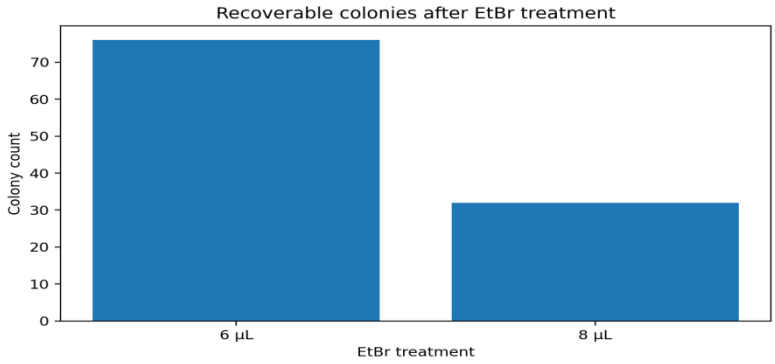

Figure 10. Recoverable colony counts reported for the enumerated EtBr treatments (6 and 8 µL). Lower treatments were recorded as lawn growth and therefore are not assigned invented colony counts.

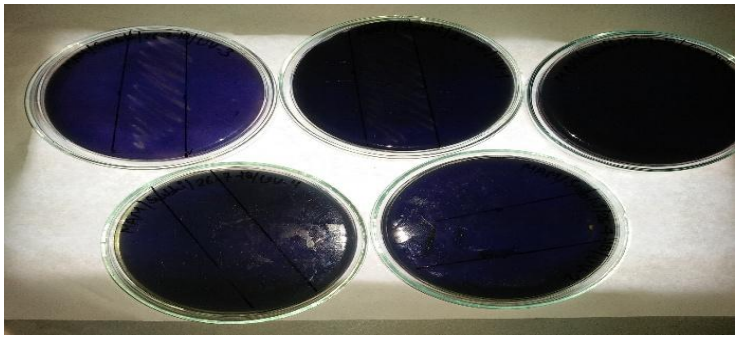

Figure 11. Plate-based screening image associated with mutagenesis and starch hydrolysis. Image reproduced from the supplied experimental record.

Growth kinetics

The supplied growth data show an increase in OD620 from 0 at inoculation to 0.22 at 24 h and 0.33 at 48 h, followed by a decline to 0.25 at 72 h. The pattern suggests that the culture reached its highest measured turbidity at 48 h under the stated conditions. This observation is useful for interpreting the subsequent 48-h fermentation experiment because the production stage was conducted for the same nominal duration.

Time (h)	OD620
0	0.00
24	0.22
48	0.33
72	0.25

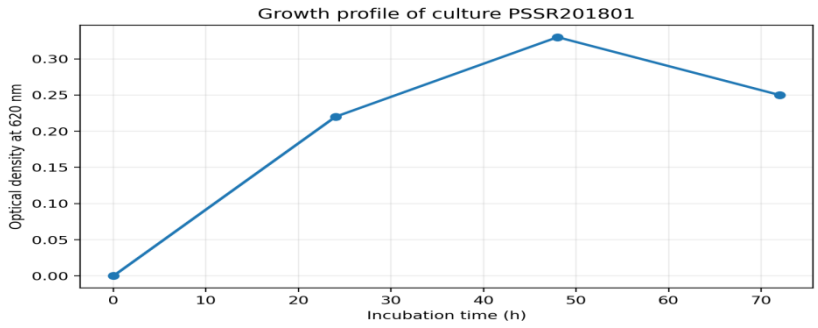

Figure 12. Growth profile of PSSR201801 based on OD620 values supplied in the original experimental record.

Enzyme production and purification outcomes

The experimental records describe production of amylase by submerged fermentation using parent, UV-mutated and chemically mutated cultures. The selected cultures were incubated for 48 h, after which culture supernatants were used for crude enzyme recovery and DNS assay. The source material states that mutagenic treatment enhanced amylase production and that the UV-treated culture showed a particularly strong response. However, the supplied files do not provide a complete table of absorbance values, standard-curve equation, enzyme units, specific activity, total protein, purification fold or recovery percentage. These missing quantitative fields prevent a rigorous numerical comparison among the parent and mutant strains.

Figure 13. Representative plate image from the mutant screening workflow. Image reproduced from the supplied experimental record.

Partial purification

Ammonium sulfate precipitation followed by dialysis yielded a partially purified enzyme preparation. The strategy is suitable as an initial concentration and desalting step before chromatographic purification. The supplied record reports that enzyme activity was re-estimated after dialysis. A complete purification table was not available, so the present manuscript does not assign a purification fold or recovery value.

Figure 14. Dialysis treatment used during partial purification of the crude amylase preparation. Image reproduced from the supplied experimental record.

DISCUSSION

The experimental sequence demonstrates a classical but still relevant strategy for microbial enzyme improvement: environmental isolation followed by phenotype-based selection and random mutagenesis. The identification of an amylolytic Bacillus from rhizospheric soil is consistent with the broad ecological distribution of Bacillus and the established importance of bacterial sources for extracellular amylase production. Microbial α-amylases are attractive industrial catalysts because they can be produced by fermentation and their characteristics can be adapted through strain selection and process engineering (Pandey et al., 2000; Sivaramakrishnan et al., 2006).

The UV data show a monotonic decline in colony recovery as exposure duration increased. This pattern is expected for a mutagenic stress in which increasing exposure can reduce viability while generating a spectrum of surviving variants. The study's selection of a 20-min-derived candidate is therefore biologically plausible, but the optimal mutagenic exposure should not be defined from colony survival alone. An industrial strain-development program must balance mutation frequency, viability, phenotypic diversity and genetic stability. Excessive lethality can reduce the size of the recoverable mutant library, whereas insufficient stress may produce too few useful variants.

The EtBr experiment similarly demonstrates selection pressure, although its reporting is less suitable for quantitative dose-response analysis because the two lower treatments were recorded as lawn growth. The enumerated values at 6 and 8 µL show a decrease in recoverable colonies, suggesting increasing stress at the

higher treatment. Because EtBr is a potent laboratory hazard, its historical use in mutagenesis should be accompanied by strict institutional safety controls and appropriate waste disposal.

The use of starch-hydrolysis screening followed by DNS assay is a strength of the workflow. Plate clearing is rapid and inexpensive, whereas DNS provides a quantitative biochemical endpoint. The combination reduces the number of cultures requiring liquid fermentation while retaining a more objective production measurement at the later stage. Classical DNS methodology remains a standard approach for measuring reducing sugars released during amylolytic reactions (Miller, 1959).

Interpretation of the growth profile

The PSSR201801 growth curve reached its highest measured OD620 at 48 h. The subsequent reduction at 72 h may indicate entry into a stationary or decline phase, although OD alone cannot distinguish reduced growth from cell lysis, aggregation or other physiological changes. The 48-h point is therefore consistent with the fermentation duration used in the production experiment, but it should not be interpreted as proof that 48 h is the absolute optimum for enzyme productivity. Enzyme production and biomass accumulation can peak at different times.

Relationship between mutagenesis and enzyme productivity

The central claim of the supplied study is that mutagenesis enhanced amylase production. This interpretation is consistent with the broader literature, where UV and chemical mutagenesis have been used to obtain higher-producing Bacillus variants. A classic study reported a stable B. subtilis mutant with approximately five-fold higher α-amylase secretion than its parental strain after successive mutagenic selection (Shah et al., 1989). More recent work has also demonstrated that UV mutagenesis can improve α-amylase productivity and enzyme properties in B. subtilis (Khelil et al., 2022). These studies support the conceptual basis of the present experiment while emphasizing that each mutant must be validated independently.

A key limitation of the present dataset is the absence of raw quantitative enzyme-production values. The supplied abstract states that 20 min of UV exposure doubled enzyme activity, but the corresponding numerical measurements are not included. It would be inappropriate to convert that statement into a fabricated activity value or to calculate statistical significance. Instead, the result should be treated as a preliminary observation requiring replication.

Industrial implications

If the selected mutant phenotype is stable and reproducible, improved amylase productivity could lower the volumetric cost of enzyme manufacture. However, industrial suitability requires more than higher activity. The enzyme must be characterized for optimum pH and temperature, thermal stability, substrate specificity, tolerance to process additives, storage stability and compatibility with the intended application. The supplied study reports optimum growth/production conditions around 37 °C and pH 4, and notes effects of Mg^{2+}, SDS and EDTA in the source discussion, but these observations are not supported by a complete numerical characterization dataset in the supplied files.

Methodological strengths and limitations

Aspect	Strength	Limitation / required improvement
Isolation	Environmental source and defined isolation workflow.	Single reported sampling location; broader ecological sampling would improve generalizability.
Screening	Rapid starch-hydrolysis plate assay.	Zone size is not equivalent to enzyme units; quantitative validation is required.
Mutagenesis	Two independent mutagenic	Replicate-level survival and mutation-frequency

	approaches were explored.	data were not reported.
Growth analysis	OD620 measurements at multiple time points.	No replicate values or error estimates were supplied.
Enzyme assay	DNS assay provides a quantitative biochemical endpoint.	Raw OD540 values and standard-curve equation were not supplied.
Purification	Precipitation plus dialysis is a reasonable initial downstream step.	No purification fold, yield or specific activity was reported.
Strain identity	Morphological/taxonomic identification was reported as B. subtilis.	Molecular confirmation such as 16S rRNA sequencing was not provided.
Statistics	Phenotypic comparisons were made.	No replicate-based statistical analysis was available in the supplied records.

Recommended next-stage validation

A PhD-level continuation should first establish the genetic and phenotypic stability of the selected mutants. Each candidate should be independently cultured across multiple generations and re-assayed for amylase activity. Molecular identification should be strengthened using 16S rRNA gene sequencing and, if feasible, whole-genome or targeted sequencing to identify mutations associated with improved production. Enzyme production should be quantified using biological replicates, with results reported as mean ± standard deviation and analyzed using appropriate statistical models.

Process optimization should then examine carbon and nitrogen sources, pH, temperature, inoculum size, agitation and fermentation time using statistically designed experiments rather than sequential one-factor optimization alone. Response Surface Methodology or other design-of-experiments approaches can identify interactions among variables and reduce experimental burden. The final strain should be evaluated at increasing fermentation scale, because oxygen transfer, mixing and heat removal can alter microbial physiology during scale-up.

CONCLUSION

The present study establishes a coherent strain-improvement workflow for enhanced α-amylase production by a Bacillus isolate recovered from rhizospheric soil near Meerut College. PSSR201801 was selected following isolation, purification and starch-hydrolysis screening. UV and EtBr treatments generated mutant populations from which candidate amylolytic colonies were recovered. UV exposure reduced recoverable colony numbers progressively from approximately 220 at 5 min to 45 at 20 min, while enumerated EtBr treatments yielded 76 colonies at 6 µL and 32 at 8 µL. The growth profile of the parent culture reached a maximum measured OD620 of 0.33 at 48 h, supporting the use of a 48-h fermentation interval in the production workflow.

The study's most important biological finding is the reported improvement in amylase production following mutagenesis, with the source record specifically stating enhanced activity after 20 min of UV exposure. The work also demonstrates the practical integration of plate screening, submerged fermentation, DNS-based reducing-sugar estimation, ammonium sulfate precipitation and dialysis. These steps constitute a useful foundation for developing a higher-producing industrial enzyme strain.

At the same time, the present dataset should be considered a preliminary strain-screening study rather than a fully validated quantitative production study. The absence of replicate enzyme-activity data, raw absorbance values, standard-curve equations, protein measurements and statistical analyses limits the strength of numerical claims. The next phase should therefore focus on reproducibility, molecular identification, genetic stability, rigorous enzyme characterization and statistically designed fermentation optimization.

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