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RESEARCH ARTICLE
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Biotechnological Application of Pectin-Degrading Enzymes from A New Strain of *Penicillium cyclopium*

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ABSTRACT

Pectin-degrading enzymes are key biocatalysts in various agro-industrial processes, yet fungal pectin lyases remain underutilized due to limited strain productivity and insufficient alkaline stability. This study aimed to isolate a high-activity pectin lyase-producing strain of *Penicillium cyclopium*, optimize its physiological conditions for enzyme production, and evaluate the potential application of its enzymes in synthetic detergent formulations. Screening of fungal cultures was performed on high-esterified D-galacturonan at pH 8.5–10. Immobilized cultivation techniques were used to enhance biosynthesis. Physiological parameters such as carbon and nitrogen sources were optimized under batch and semi-continuous modes. The enzyme preparation was obtained via ethanol precipitation and characterized by pH and temperature optima, substrate specificity, and stability in detergent-like alkaline conditions. A novel strain, *Penicillium cyclopium* 2-11, was selected based on its high pectin lyase activity, which exceeded the parental culture by 6.6-fold. Optimal enzyme production was achieved using fructose and ammonium chloride as primary carbon and nitrogen sources, respectively. The enzyme preparation exhibited maximum activity at pH 10–11 and 50–60°C, with high specificity for both highly and lowly esterified D-galacturonan. The enzymes-maintained stability under alkaline conditions (pH 12) at elevated temperatures and improved detergent performance by 25%, while reducing detergent usage by half. The immobilized strain *Penicillium cyclopium* 2-11 represents a promising biotechnological source of pectin lyases with potential for eco-friendly, cost-effective applications in the detergent industry and other agro-industrial sectors.

Keywords: *Penicillium cyclopium*, Detergent biotechnology, Fungal enzymes, Agro-industrial application, Biocatalyst optimization.

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INTRODUCTION

Pectin is a complex plant-derived polysaccharide composed of a galacturonic acid (GalA) backbone with neutral sugar side chains branching from it (Bazargaliyeva et al., 2024). The structural characteristics of pectin can vary considerably depending on the extraction method and subsequent chemical modifications, leading to differences in monosaccharide composition, glycosidic linkages, and degrees of methylation and acetylation (Li et al., 2023). The present study focuses on the biosynthesis of pectin lyase (PL) enzymes and their potential application in enhancing the cleaning efficiency of synthetic detergents (SD). Pectin-degrading enzymes, particularly polygalacturonases and pectin lyases, have garnered significant interest due to their ability to depolymerize plant polysaccharides that are abundant in the cell walls and intercellular spaces of higher plants (Safuani, 2005). These enzymes (polygalacturonases and pectin lyases) are also used in various industries; polygalacturonases, which

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hydrolyze the glycosidic bonds between galacturonic acid residues in pectin, are used in food processing, biorefineries, biofuel, and textile industries (Kumar et al., 2021; Serra et al., 2024). Pectin lyases, on the other hand, whose action is based on catalyzing the transelimination of pectin, breaking the glycosidic link without directly hydrolyzing it, are less often used and are currently being researched for use in food technology and bioprocessing (Yadav et al., 2023).

Although both enzyme classes act on the same substrate and the same α-1,4 linkage in pectin compounds, they differ in their cleavage mechanisms and optimal pH ranges: polygalacturonases function via a hydrolytic mechanism under acidic to neutral pH, whereas pectin lyases employ a β-elimination mechanism under strictly alkaline conditions. Alkaline pectin lyase is an industrially important pectinolytic enzyme that catalyzes the cleavage of highly esterified pectin through a β-elimination mechanism, producing unsaturated oligogalacturonides. Over the years, research has shown that alkaline PL were beneficial for processes such as ramie degumming (which is the process of separating gummy cellulose materials prior to the downstream spinning process) due to their thermostability (Zhou & Wang, 2021; Zhou et al., 2022). Li et al. (2022) demonstrated the modification of alkaline PL by using fragment replacement. The new product displayed increased catalytic activity, optimum pH and temperature, and also a superior ability to cleave methylated pectin. In addition to their current application in textile and pulp industries, Sheladiya et al. (2022) explained that alkaline PL can also be utilized in juice clarification. Pectin hydrolases produced by various microorganisms have been well studied and are extensively used in the juice and wine industry, animal feed production, and flax retting (Kumar et al., 2002; Timorshina et al., 2025). In contrast, pectin lyases remain relatively poorly investigated and have yet to find wide practical application with most of its application being in the developmental phase, this is due to the complexity of their substrate (Li et al., 2022; Yüksel et al., 2024).

In recent years, a promising new field has emerged globally – the use of enzymes as active additives in synthetic detergents. This approach is based on their ability to break down complex compounds into simpler ones, which are easily removed with water. Alpha-amylase is commonly used in SD formulations to eliminate carbohydrate-based stains. However, α-amylase alone is insufficient to remove carbohydrate contaminants, especially those involving pectin-rich residues. Such stains are frequently found on clothing and textiles of individuals working in the food industry, catering services, and household environments. Detergent enzymes, respectively, refer to proteases, amylases, lipases, cellulases, pectate lyases, and mannanases, which possess the ability to break down proteins. Recent advancements in detergent enzymes have highlighted the existence of cold-active detergent enzymes. Al-Ghanayem and Joseph (2020) explained that for cold regions, psychrophiles, which are cold-active enzymes with high catalytic activity and stability under extreme conditions, can serve as appropriate eco-friendly and cost-effective additives in detergents. They further explained that by altering the chemo-, regio-, or stereo-selectivity of the protein region of these enzymes, they can increase their activity and specificity. Maghraby et al. (2023) explained that wool-based fabrics are not harmed by detergent enzyme immobilization, which also increases cleaning effectiveness and maintains enzyme catalytic activity. Natural protein fibers are hydrolyzed by proteases, which lowers the quality of clothing. Studies also prove that free enzyme samples only kept 37% of their initial tensile strength, whereas immobilized protease kept 76%. Wool fibers were not harmed by the immobilized enzyme either. After an hour at 60°C, the nanoenzyme made with immobilized protease still had 63.6% activity.

To effectively hydrolyze pectin-based contaminants from fabric fibers, we propose the incorporation of pectin lyase enzymes (PLs) in SD formulations to enhance their cleaning efficacy. By utilizing the substrate specificity of PLs and their optimal activity under alkaline pH (in contrast to pectin hydrolases that operate in acidic or neutral conditions), it is possible to develop highly active, targeted enzymatic preparations that open a new application niche. However, the development of such formulations presents certain challenges, including the lack of available high-yield producers of individual pectin lyases and undefined conditions for their production.

The development of new detergent formulations with biologically active additives effective in removing various contaminants is an urgent task that requires both fundamental and applied research. Given the high daily demand for detergents and their high production cost, it is crucial to develop domestic, cost-effective SDs based on modern biotechnological approaches, including continuous cultivation of high-activity enzyme-producing strains in an immobilized state (Kumar et al. 2003). The inclusion of pectin lyase enzymes in detergents is unpopular with the most recent mention of this process dating back to a withdrawn patent in 1996, from that patent it is understood that a wide variety of body, plant, and fruit-based stains may be removed by pectin lyase enzymes, particularly alkaline ones, which also improve the detergent compositions' actual item cleaning profile. This aligns with the study of Maurer (2024). Madhu (2022) reported that immobilization of enzymes was key to maintaining sustainability in the textile industry since it allows for the effective recovery and reuse of expensive enzymes, as well as better enzyme function through improved stability in both storage and operating settings. He also added that immobilization has the potential to provide additional functionalities to textiles. Immobilized enzymes are also applicable in textile bioscouring processes (Taleb et al., 2021). Jayakumari et al. (2024) explained that in comparison to conventional scouring methods, textile bioscouring saved around 42% of energy, 50% of water and 33.33% of time. Furthermore, Kundu et al. (2020) provided an exposé on in situ immobilization, which is an emerging process that allows for the production of active enzymes and carrier materials simultaneously by bioengineering microbes. They further explained that in situ immobilization is cost-effective and allows for the production of soft materials. Recent studies have confirmed the application of alkaline PLs in textile bioscouring and detergent (Aggarwal et al., 2020; Sharma et al., 2022; Shoily et al., 2023).

Pectin lyase enzymes (PLs) represent a unique group of biological catalysts that cleave α-1,4-glycosidic bonds between D-galactopyranosyluronic acid residues in pectins via a β-elimination mechanism. Unlike pectin hydrolases, they function under alkaline pH conditions and do not require water as a reactant. The discovery of these enzymes is credited to Albersheim, who, while studying a "pectinase" preparation derived from Aspergillus niger, identified a non-hydrolytic enzymatic activity and named it pectin lyase or pectin trans-eliminase (Ruadze et al. 2001).

The scientific interest in this class of enzymes lies, first, in their distinctive non-hydrolytic cleavage mechanism, which involves hydrogen atom transfer at the C-5 position and formation of a double bond between C-4 and C-5 at the non-reducing end of the galacturonic acid residue. Second, these enzymes offer potential for solving a range of practical biotechnological problems. The novelty of this study lies in the production of Penicillium cyclopium 211 (a biocatalyst for the production of alkaline pectin lyase (PL)), through immobilization and prolonged cultivation. Beyond a 6.6-fold increase in activity, its novelty includes remarkable operational stability at pH 12 and 50–60°C, which are typical of harsh detergents and the temperatures at which the majority of fungal PLs deactivate. The immobilization technique yielded significant financial gains, eliminating the need for culture fluid filtration and reducing inoculum usage by a factor of 12. Additionally, the strain's unique morphological and physiological characteristics point to distinct genetic traits, and its ITS barcode serves as a reliable taxonomic identifier. *P. cyclopium* 2-11 is a superior and commercially viable enzyme source due to its strong enzymatic qualities, process economics, and genetic uniqueness.

Pectic substances are widespread in the cell walls of higher plants, particularly in the middle lamella, where they serve structural and adhesive roles (Martynenko et al., 2004). Young growing tissues are especially rich in pectins, predominantly in the form of insoluble protopectin, which becomes soluble as the tissue matures.

Homogalacturonan (HG) is a linear polymer of galacturonic acid residues, often methyl-esterified, and periodically interspersed with rhamnose residues. Rhamnogalacturonan I (RG-I) consists of alternating galacturonic acid and rhamnose units, bearing side chains of arabinans, galactans, and arabinogalactans, giving the molecule a "hairy" structure. Rhamnogalacturonan II (RG-II) is a more complex polysaccharide with unique side chains containing fructose, xylose, and apiose, capable of forming borate cross-links (Dessouki et al., 2001; Szczesna et al., 2001).

The structural diversity of pectic polysaccharides determines the specificity of different pectin lyases, enabling selective degradation of plant cell wall components.

The mechanical properties of fruits and vegetables depend on the degree of pectin esterification. Unripe fruits contain protopectin, which converts to soluble pectin during ripening, resulting in tissue softening. Calcium salts promote stabilization by forming pectinates. Pectins are soluble in water and polar solvents; their solution viscosity depends on molecular weight, pH, and temperature. Alkaline and acidic treatments can cause depolymerization: bases de-esterify methyl groups, especially at high temperatures, while acids hydrolyze glycosidic linkages, notably in arabinans. Pectins are unstable to sterilization at neutral pH and sensitive to metal ions and radiation. Their ion-exchange properties are due to carboxyl groups.

Recent studies have shown that the cell wall in plants can mediate the recognition of surrounding cells, and fer receptor kinases play key roles in reproductive success. Pectin is involved in the signaling link between FER and nitric oxide (NO), and genetic changes that reduce its level increase the incidence of polyspermy. Pectin also participates in the accumulation of NO in the filiform apparatus, triggered by pollen tube arrival at ovules. The cell wall sensor FERONIA (FER) senses salinity through its pectin-binding extracellular domain, leading to increased cytosolic and mitogen-activated protein kinase 6 (MPK6) function. Pectin is also involved in signaling processes during interactions with the environment, such as salt stress, cold stress, and pathogen infection (Shin et al., 2021).

Enzymatic degradation of pectins is carried out by various pectin lyases, including polymethylgalacturonate lyase (PMGL), endo- and exo- polygalacturonate lyases (PGLs), and oligogalacturonate lyases (OGLs). PMGLs act on highly esterified pectins, with optimal activity at pH 5–6. Endo-PGLs cleave pectates with varying degrees of esterification, with polygalacturonates being the most active substrates (Miyairi et al., 2002). Exo-PGLs release unsaturated monomers from the reducing end, while OGLs are effective on short-chain substrates. Most reported pectin lyases exhibit maximal activity around pH 8–10, whereas the present strain (*P. cyclopium* 2-11) demonstrates optimal activity at slightly higher alkalinity (pH 10–11), which is advantageous for detergent applications.

Pectin lyases are produced by microorganisms, including Erwinia, Bacillus, Pseudomonas species, and Penicillium fungi (Timorshina et al., 2025). Both endo- and exo-forms have been identified in fungi, with activity across broad pH and temperature ranges (Nyanikova et al., 2002; Shim and Yang, 2002). Gül et al. (2024a) reported that pectin lyases are commonly industrially synthesized by molds and exhibited peak activity at 50°C. In Erwinia, multiple isoforms of pectate lyase and a single polygalacturonase have been described. These enzymes degrade plant cell walls and have found applications in the food industry. Enzyme secretion depends on medium composition, particularly the presence of sodium pectate and calcium ions.

To increase enzyme yield, immobilized cells are employed. Immobilization – anchoring microbial cells onto carriers – enhances stability, productivity, and enables reuse. Carriers include calcium alginate, agarose, chitosan, and polyacrylamide, among others. Methods include gel entrapment, adsorption, and chemical or physical binding. Immobilized cells are used to produce enzymes, organic acids, and in wastewater treatment. The best results have been obtained using gels and membrane-based systems. The biosynthesis of pectin lyases is influenced by cultivation conditions, particularly the sources of carbon and nitrogen. Pectin, galacturonic acid, and their derivatives serve as inducers. Some strains exhibit inducible regulation, while others are constitutive. Catabolite repression also affects synthesis: glucose may inhibit enzyme production (Guan et al., 2002). Optimal biosynthesis has been achieved using pectin as the carbon source and ammonium sulfate as the nitrogen source. Calcium ions activate enzymes, while chelating agents like EDTA inhibit them.

Selection of high-activity strains is crucial for efficient enzyme production (Admanova et al., 2024). Methods include UV mutagenesis, chemical mutagenesis, and isolation of spontaneous mutants with enhanced activity (Mikhailova et al., 2001). Notable examples include *Chaetomium thermophile, Streptomyces lavendulae*, and *Gongronella butteri*. Strains are selected based on thermostability, productivity, and stress resistance. Intrapopulation variability is harnessed as a resource for strain improvement. Immobilized cultures also exhibit enhanced productivity and resilience during extended cultivation. In summary, pectin lyases are promising enzymes for biotechnology. They can be produced using both free and immobilized cells. Cultivation conditions, medium composition, and strain selection are key factors influencing efficiency. Advances in strain selection and process optimization enable the development of high-yield systems for pectin lyase production.

The objective of this study was to isolate an active producer of pectin lyase enzymes from both culture collections and newly obtained microbial isolates, to select a highly productive strain through immobilized cultivation, to develop a targeted biosynthetic strategy for maximizing enzyme production, and to evaluate the potential application of these enzymes in enhancing the cleaning efficiency of synthetic detergents. To achieve this objective, microorganisms exhibiting strong pectin lyase activity were isolated from both existing culture collections and newly obtained samples, with particular focus on strains capable of utilizing highly esterified D-galacturonan as a substrate under alkaline conditions (pH 8.5–10). The nutritional physiology of the selected strain was investigated to identify optimal carbon and nitrogen sources for maximum enzyme biosynthesis during batch cultivation. To further enhance enzyme production, the active strain was immobilized on a suitable carrier, and conditions influencing the variability and productivity of the immobilized culture were systematically examined to generate highly active variants. The nutritional and physiological properties of the high-activity strain were further analyzed to optimize biosynthesis of the target enzymes. Subsequently, the pectin lyase enzymes were isolated and their physicochemical properties characterized. Finally, the obtained enzymes were applied for the removal of pectin-containing carbohydrate stains, demonstrating their potential in improving the overall cleaning performance of synthetic detergents.

MATERIALS & METHODS

The experimental work was conducted between 2022 and 2024 at the laboratories of K. Zhubanov Aktobe Regional University under controlled conditions. In this study, pectin lyase enzymes (PLs) were proposed and applied for the first time as active agents in synthetic detergents (SD) for the hydrolysis of pectin-containing contaminants. An active microbial producer of these enzymes was isolated, and the conditions that enhance their biosynthesis were identified. Immobilization of the culture enabled the selection of a high-activity variant. PL enzyme preparations were obtained and their physicochemical properties – such as pH and temperature optima, and substrate specificity – were characterized, supporting their potential use in SD formulations. As a result of studying the population of immobilized cells, 15 colony types were identified. Among them, the most active strain was selected – *P. cyclopium* 2-11, which demonstrated enzyme activity 6.2–6.6 times higher than the parental culture. The applied selection method resulted in a new strain differing from the original not only in morphology, structure, and enzyme productivity but also in nutritional physiology (Martynenko and Gracheva, 2003). An enzyme preparation was isolated from the culture liquid of immobilized *P. cyclopium* 2-11 using ethanol precipitation. The preparation was subjected to physicochemical characterization, including determination of pH and temperature optima and substrate specificity (Desimone et al., 2002). From newly isolated microorganisms capable of growing on a specific substrate – highly esterified D-galacturonan at pH 8.5–10 – a novel culture was identified as the most active PL producer, forming a clearing zone of up to 10–11mm. It was taxonomically classified as *P. cyclopium*. Primary screening of PL-producing strains was carried out in Petri dishes on Czapek-Dox agar medium containing highly esterified D-galacturonan as the specific substrate. The ability of cultures to produce pectin lyases was evaluated by measuring the diameter of the clearing zones in the substrate after 5–7 days of incubation. Fungal cultures were cultivated and maintained on Rolen's slants under standard conditions for 4–5 days at 28–30°C, and stored at 3–5°C. The Czapek medium was used to determine nutrient requirements for *P. cyclopium*. The initial composition of the medium included (%): $NaNO_3$ – 0.15% N, sucrose – 2%, KH_2PO_4 – 0.1%, $MgSO_4$ – 0.05%, KCl – 0.05%, $FeSO_4$ – 0.001%.

Various mono-, di-, and polysaccharides, as well as polyhydric alcohols, were tested as carbon sources (Serralha et al., 2001). Mineral nitrogen sources were normalized to 0.15% total nitrogen. Organic nitrogen sources were also evaluated. All media were sterilized at 0.5 atm for 30–40 minutes. In the culture fluid, pH, enzyme activity (pectin lyases and others), and dry biomass (dried to constant weight at 105°C) were determined to calculate culture productivity.

Cultivation of P. cyclopium PL-Producing Strain

The taxonomic identification of the most active strain was performed using identification guides. *P. cyclopium* was cultivated both in immobilized and free form under submerged fermentation conditions (Shigaeva et al., 2004). The inoculum consisted of a spore suspension from a pure culture, applied at 2% of the medium volume, containing 170,000–190,000 spores permL (Ding et al., 2003). Immobilization was achieved via adsorption by introducing the spore suspension into the nutrient medium containing an adsorbent material. Immobilized cultivation was conducted on a fixed substrate (belt material) immersed in the medium and attached to a fixed rod. A specialized laboratory apparatus (Author's Certificate No. 1047954) was used for immobilization. After agitation on a shaker, spores adhered to the support surface, forming a film that developed into a filamentous-sponge-like mycelial structure. Free mycelium was separated from the culture fluid by Büchner funnel filtration, whereas immobilized biomass did not require this step.

Enzyme Activity Assays: PMGL, PGL, PG, α-Amylase, Proteases

(A) Determination of PMGL and PGL Activity

In this study, the following abbreviations are used consistently:

PMGL – polymethylgalacturonate lyase (acts on highly esterified pectin);

PGL – polygalacturonate lyase (acts on low-esterified pectin);

PG – polygalacturonase (hydrolase, used as comparative enzyme);

The term pectin lyase (PL) refers collectively to PMGL and PGL activities.

The action of PMGL and PGL on pectin substances yields unsaturated products containing a C4–C5 double bond at the non-reducing end of the galacturonic acid residue, with hydrogen transfer to an adjacent residue. Enzyme activity was determined spectrophotometrically by measuring UV absorbance of the reaction products at 235 nm, following the method of Albersheim. The substrate for PMGL was highly esterified D-galacturonan; for PGL, less esterified D-galacturonan. The reaction mixture consisted of 2.5mL of 1% substrate solution (in Tris-HCl buffer, pH 8.5–10) and 0.5mL of enzyme solution. The mixture was incubated at 37°C. After 1 hour, 1mL was withdrawn, diluted tenfold, and absorbance was measured at 235 nm.

(B) Determination of Polygalacturonase (PG) Activity

Polygalacturonase belongs to the class of hydrolases and catalyzes the hydrolysis of α-1,4-glycosidic bonds between non-esterified galactopyranosyluronic acid residues in pectic substances. It does not act on esterified glycosidic bonds. The action of PG results in a decrease in the viscosity of sodium pectate or low-esterified pectin solutions and an increase in the concentration of reducing groups in the incubation mixture. PG activity was measured via viscosity reduction using the Whittaker method with modifications, utilizing an Ostwald viscometer. The reaction mixture included 5mL of 1% pectic acid solution, 0.5mL of 0.1M acetate buffer (pH 4.6), and 0.5mL of enzyme solution or culture fluid. Measurements were conducted in a water thermostat at 40°C at 2-minute intervals for 6 minutes. One unit of activity was defined as the amount of enzyme that results in an increase of the reciprocal of relative viscosity by one unit per minute.

(C) Determination of α-Amylase Activity

Amylolytic activity (AA) was determined based on the extent of starch hydrolysis as a function of enzyme quantity (Neklyudov and Denyakina, 2004). The degree of hydrolysis was assessed by the decrease in iodine staining of starch, measured photocolorimetrically. One unit of α-amylase activity was defined as the amount of enzyme that hydrolyzes 1g of starch in 1 hour at 30°C under standardized conditions, yielding 30% substrate conversion. According to IUB enzyme unit definitions, α-amylase activity can also be expressed in micromoles of hydrolyzed starch, using the assumed molecular weight of a glucose residue in starch (162g/mol). One international unit (IU) of α-amylase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1μmol of starch per minute. Optical density was measured in cuvettes (path length 1 cm) with a red light filter (λ = 656 nm). Absorbance values were obtained for the control solution (D1, corresponding to 0.1 g of starch) and the experimental solution (D2, indicating the amount of hydrolyzed starch and dextrins). Activity was calculated using standard formulas.

(D) Determination of Proteolytic Enzyme Activity

Proteolytic activity was determined using a modified Anson method following GOST 20264.2-74. The method is based on the hydrolysis of sodium caseinate by proteases, followed by inactivation and precipitation of undigested protein with trichloroacetic acid (TCA). Color intensity of the reaction mixture was measured using a photoelectric colorimeter against a control sample at 630–670 nm in 10 mm path length cuvettes.

Study of Variability in Immobilized *P. cyclopium* Culture

Variability of the immobilized *P. cyclopium* culture was assessed by examining structural-morphological characteristics, enzyme production capacity, and changes in nutritional physiology. Initial mycelial samples were taken 6–7 days post-immobilization and again after extended cultivation (25–30 days). Samples were collected from both surface and internal regions of the immobilized structure. Monospore suspensions were prepared by adding 5–9mL of sterile distilled water to test tubes containing mycelial fragments, including single spores and spore chains. Suspensions were filtered through sterile multilayer filter paper into sterile tubes. The spore suspension was diluted to concentrations of 1:1000, 1:10,000, 1:100,000, and 1:1,000,000, and 0.1mL of each was plated on solidified agar medium in Petri dishes. The spores were evenly spread using a sterile glass spatula, and plates were incubated for 7–9 days at 28–30°C. Post-incubation, colony counts were recorded. Morphologically distinct variants were assigned individual codes and cultured in liquid nutrient media with adsorption substrates. Pectin lyase activity in the culture fluid was then determined. The most active variants were further cultivated under continuous growth conditions and re-evaluated for enzyme production levels.

Isolation and Characterization of Pectin Lyase Enzymes

Pectin lyase enzymes were isolated from the culture fluid of *P. cyclopium* 2-11 by ethanol precipitation at a ratio of 1:4. Pectic acid and rhamnogalacturonans of various esterification degrees were used to assess substrate specificity. pH and temperature optima were determined by measuring reaction rates in buffer solutions at varying pH values and temperatures. Both the culture fluid and ethanol were pre-cooled to 2–4°C. After rapid mixing of the ethanol with the culture fluid (3–5min), the mixture was left to settle for 10–15min. The precipitate was separated by decantation and filtered using a Büchner funnel. The residue was dried in a vacuum desiccator, ground in a porcelain mortar, and analyzed for yield and enzymatic activity. Physicochemical properties including pH and thermal stability, and the impact of alkaline detergent conditions on enzyme activity, were investigated.

Evaluation of Pectin Lyase Enzyme Performance

The effectiveness of enzymatic treatment for carbohydrate-based stains on textile fibers was evaluated under both laboratory and industrial laundry conditions (Kazakh National University named after al-Farabi).

Tests were performed on 100% cotton white table linen (surface density 150±5g m^{-2}) cut into 10 × 10 cm samples. Each sample was stained with 1mL of a pectin-rich mixture prepared from equal parts of apple, beet, and berry juices containing 1% (w/v) natural citrus pectin and 0.5% sucrose, then air-dried for 24h. Washing was carried out in a rotary laboratory washer at a bath ratio of 1: 20, agitation speed 100rpm, and temperature 50–60°C for 60min, followed by rinsing in distilled water and air-drying. The enzyme preparation was added at 0.5% w/w of detergent, corresponding to approximately 140UL^{-1} total pectin-lyase activity. Washing performance was evaluated on a five-point visual scale (1 = no cleaning, 5 = complete cleaning) calibrated using colorimetric reflectance values; a residual color difference of $\Delta E < 3$ between cleaned and unstained fabric was considered "complete stain removal." Representative pre- and post-washing images were recorded under identical lighting, and instrumental reflectance data are available upon request.

Microscopic Observations

Microscopic examination of both free and immobilized cells was performed using a light microscope (MBI-3) at 300× magnification. Samples were fixed with Carnoy's solution and stained with diluted methylene blue (1:1000).

Statistical Analysis of the Data

All statistical computations were carried out using OriginPro 2023. All assays were performed in triplicate independent repetitions (n = 3). Data are reported as mean±standard deviation (SD). Each table and Fig. now indicates sample size (n) and statistical significance. To ensure the reliability of the results, all obtained data were subjected to statistical analysis. The standard deviation or variance was calculated using the following formula:

$$\sigma = \sqrt{\frac{\sum (\chi_i - \bar{\chi})^2}{n-1}};$$

Where, n-1- degrees of freedom

X_i - partial arithmetic mean

$\bar{X}$ - mean

The arithmetic mean or the standard error of representativeness is calculated using the following formula:

$$\bar{m} = \frac{\sigma}{\sqrt{n-1}}$$

The coefficient of variation is denoted by CV, and is calculated as follows:

$$CV = 100\frac{\sigma}{\bar{\chi}}\%$$

The absolute frequency of individual variants in a statistical population is determined by the formula:

$$P = \frac{CV}{\sqrt{n}};$$

$$Hcp = \frac{\sigma \cdot t_{0.05}}{\sqrt{n}};$$

Where $t_{0.05}$ is the Student's t-value at a significance level of 0.05, as used in biological sciences.

The reagents used in our experiments were purchased from the companies *Veld*, *Pharmakhimiya*, and *Almatykhimreaktiv*.

RESULTS AND DISCUSSION

This study demonstrates *P. cyclopium* 2-11 high potential as a biotechnological producer for pectin lyase (PL) enzyme. The biocatalysis of pectin-containing substrates, which has many industrial applications, especially the detergent industry, is made possible by the isolation and optimization of this strain. The results indicate that the strain is a viable option for large-scale industrial applications due to its increased productivity as well as the effectiveness of immobilization techniques in increasing enzyme output and lowering operating costs. This study aligns with recent research attempts to develop low cost to no-cost substrates for producing pectinases (Shrestha et al., 2021; Li & Tian, 2024).

Selection of an Active Producer of Pectin Lyase Enzymes

At the beginning of the study, a number of micromycete strains known as active producers of various pectin-degrading enzymes were available. These included the following strains: *Aspergillus awamori* 16, *A. niger* P, *A. niger* 355, *A. foetidus* strains 1–5, *Penicillium chryzogenum* 241, *P. chryzogenum* 245, as well as up to 14 newly isolated cultures of microorganisms capable of growing on specific substrates—highly esterified D-galacturonan at pH 8.5–10 (Table 1).

As a result of these observations, it was found that although many cultures demonstrated the ability to degrade pectin, they were unable to grow on pectin-containing substrates under alkaline pH conditions. Only one newly isolated strain, Culture No. 6, exhibited rapid growth and development at pH 8.5–10 on specific substrates, forming clearing zones of up to 10–11 mm. This occurrence is due to the fact that pectin-degrading enzymes, like pectinases, often have optimal activity and stability in a more neutral to slightly acidic pH range. Some pectinases are more active in acidic environments, while others are more active in alkaline conditions (Khattab, 2022; Gül et al., 2024b).

Morphological Characteristics

Colonies exhibited abundant powdery sporulation (Fig. 1), with a velvety texture and bluish-green coloration. On Czapek medium, after 10–12 days of incubation, colonies reached 4–5 cm in diameter and displayed radial wrinkling in the center, with a white marginal ring 1–2 mm wide. The reverse side of the colony was pale yellow. No exudate was observed. Conidiophores were straight, colorless, with a rough surface, 3–3.5μm in thickness, and formed indistinct bundles in the colony center. Each conidiophore bore 1–3 branches, measuring 15–35.5 × 2–2.5μm, with whorled metulae. Each bundle contained 3–5 sterigmata, slightly tapered toward the tip. Conidia were spherical, smooth or finely roughened, 2.3–4μm in diameter, forming short tangled chains and accumulating as spore masses. The colonies emitted a faint, indistinct odor.

Physiological and Biochemical Characteristics

The culture is aerobic, with an optimal growth temperature of 28–30°C. It effectively assimilates mono-, di-, and polysaccharides as well as polyhydric alcohols. All tested carbon sources supported constructive metabolism. Among them, fructose, glucose, and sucrose were the most efficiently utilized, resulting in biomass accumulation ranging from 0.85 to 1.01 g/100mL (Table 2). The culture demonstrates a high capacity to degrade highly esterified D-galacturonan.

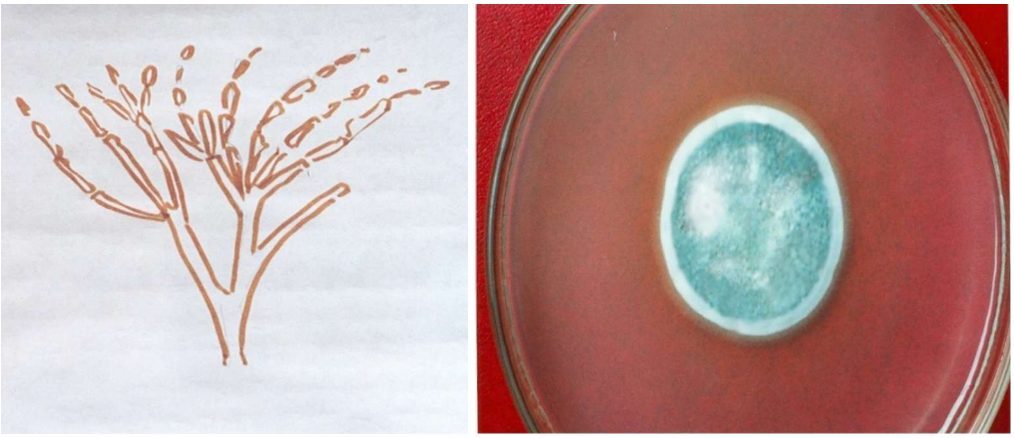
Table 1: Screening for pectin lyase enzyme producers

No	Producer Strain	Pectin Lyase Activity	Substrate Degradation Zone Diameter (mm)
1	A.awamori 16	+	0.5-0.7
2	A.niger P	+	0.7-0.9
3	A.niger 355	-	-
4	A.foetidus strain1	-	-
5	A.foetidus strain 2	-	-
6	A.foetidus strain 3	-	-
7	A.foetidus strain 4	-	-
8	A.foetidus strain 5	-	-
9	P.chryzogenum 241	-	-
10	P. chryzogenum 245	-	-
11	Culture No. 6	+	10-11

Note: (+) – clearing zone present; (–) – no clearing zone observed.

Table 2: Growth characteristics of *P. cyclopium* on various carbon sources

No	Carbon Source	Growth Character in Batch Conditions	Dry Mycelial Weight (g/100mL)	Substrate Clearing Zone Diameter (mm)
1	Fructose	Slurry-like	1.01	17
2	Glucose	Slurry-like	0.86	17
3	Sucrose	Pellets	0.85	17
4	Maltose	Pellets	0.56	14
5	Galactose	Pellets	0.66	14
6	Xylose	Pellets	0.78	14
7	Lactose	Pellets	0.52	14
8	Mannitol	Pellets	0.51	11
9	Sorbitol	Pellets	0.76	11
10	Pectin	Pellets	0.40	11
11	Starch	Pellets	0.51	11



a) Microscopic features of the culture

a) Microscopic features of the culture

Fig. 1: Morphological characteristics of the pectin lyase-producing strain *P. cyclopean.*

As a result of screening newly isolated microorganisms capable of growing on a specific substrate-highly esterified D-galacturonan at pH 8.5–10—a novel active strain was selected, forming substrate degradation zones of up to 10–11mm. The culture was identified as belonging to the *Moniliaceae* family, and its genus and species were determined as *P. cyclopium*. The culture was deposited and used in further research as the most active pectin lyase producer. These findings aligned with recent studies focused on the industrial production of pectinases. Recent studies have reported on the use of diverse penicillium species in production such as *Penicillium verruculosum, P. cyclopium,* and *Penicillium griseofulvum* (Krumova et al., 2021; Sinitsyn et al., 2021)

Semi-Continuous Biosynthesis of Pectin Lyase Enzymes by Immobilized *P. cyclopium*

When the *P. cyclopium* culture was immobilized on a flat solid support, each cell exhibited linear growth across the surface, maintaining continuous access to nutrients in the medium. During long-term cultivation, the nutrient medium was replaced every 3–4 days to maintain optimal conditions. Following each medium replacement, the culture initially formed a surface film on the support, which subsequently developed into a filamentous-sponge-like mycelial structure. Long-term cultivation of immobilized *P. cyclopium* resulted in extended biosynthesis of pectin lyase enzymes due to the use of a specially developed device. The process proceeded in several stages. As mycelial mass accumulated and aged on the carrier, enzyme biosynthesis began to decline due to reduced aeration and biomass overgrowth. To restore productivity, the entire biomass was removed from the support using the aforementioned apparatus, marking the end of the first stage, which typically lasted up to 16 days. After the removal of biomass, the second stage of biosynthesis commenced. At first, the culture fluid displayed low enzymatic activity. However, within 2–4 days, residual cells retained in the carrier pores regenerated sufficient biomass to produce enzyme levels comparable to 1.0–1.2g of young mycelium from the first stage. The subsequent dynamics repeated those of the first cycle. Under batch cultivation with free *P. cyclopium* cells, maximum enzyme production was achieved on days 3–4, yielding up to $12.9U{\cdot}mL^{-1}$ of PMGL and $15.9U{\cdot}mL^{-1}$ of PGL, with the stationary phase lasting only one day. In contrast, immobilized cultures maintained on a carrier for 44 days exhibited significantly enhanced and prolonged enzyme production, ranging from 15.8 to $26.0U{\cdot}mL^{-1}$ for PMGL and 16.0 to $28.5U{\cdot}mL^{-1}$ for PGL (Table 3). Remarkably stable enzyme activity was maintained between days 7–13 and 22–29, suggesting that the stationary phase of the immobilized culture extended for approximately 6–7 days, in contrast to the brief one-day stationary phase observed in free-cell cultures. Following this period, cell lysis occurred, leading to a gradual decline in enzymatic activity within the culture medium. During the multi-stage cultivation process, an increase in the culture's enzyme-producing capacity was observed. This growth was dependent on biomass accumulation on the carrier and the level of enzyme production in the culture fluid. As pectin lyase activity increased, so did the overall productivity of the culture. When biomass over accumulated, the enzyme-producing capacity of *P. cyclopium* declined; however, after removal of excess mycelium from the support, both PMGL and PGL production gradually increased again.

By the end of the cultivation cycle, the enzymatic activity of the culture fluid (PMGL and PGL) in the immobilized *P. cyclopium* culture increased by 1.8–2.0 times compared to the free culture (Table 3). The frequency of target enzyme production also gradually increased. While in batch culture the maximum yield of the target enzyme occurred only once, on day 4, the immobilized system allowed peaks every three days – up to 12 times In addition, during pectin lyase biosynthesis by the immobilized filamentous-sponge-like structure of *P. cyclopium*, labor costs were reduced 12-fold, as was the quantity of inoculum required. Filtration of the culture fluid was no longer necessary, and the yield of the culture fluid increased tenfold (Table 4). During the cultivation of immobilized *P. cyclopium*, several advantages over free-cell cultures were observed. These advantages stemmed from improved physiological conditions for immobilized cell associations, in which mycelial hyphae grew linearly along the carrier surface without forming pellets. This morphology allowed for optimal access to nutrients and oxygen for all cells. Thus, by adsorptive immobilization of *P. cyclopium* spores on a carrier, a filamentous-sponge-like mycelial structure was formed. Under continuous cultivation with periodic medium replacement, the immobilized mycelium produced pectin lyases for over 40 days. The enzymatic activity of culture filtrates increased from $12.9–15.9U{\cdot}mL^{-1}$ to $26.0–28.5U{\cdot}mL^{-1}$, and the culture productivity doubled, resulting in a significant improvement in volumetric efficiency.

Overall, this cultivation strategy resulted in a substantial improvement in both the efficiency and sustainability of enzyme production. The stationary growth phase and enzyme synthesis period were extended to 6–7 days, accompanied by a 1.8–2.0-fold increase in enzymatic activity and nearly a 10-fold rise in volumetric productivity. Moreover, the immobilized culture system allowed for more than 12 consecutive filtrate harvests, compared to only a single harvest in batch cultivation, while reducing labor and inoculum requirements by approximately 12-fold. Additionally, the process eliminated the need for culture fluid filtration. Collectively, these advantages highlight not only significant technological progress but also notable economic efficiency in the production of PMGL and PGL (pectin lyases) by *P. cyclopium*.

Selection of a Highly Active Variant of *P. cyclopium* 2-11 – Pectin Lyase Producer

During the cultivation of immobilized *P. cyclopium*, a number of advantages over free-cell cultures were observed. These aligns with the studies of Lapponi et al. (2021) and Tsegaye et al. (2023) who reported that Immobilization of cells allowed for increased cell density, improved stability, and enhanced productivity. They further concluded that this process made cells suitable for continuous operation, reduce downstream processing costs, and facilitate cell re-use. Other advantages included

Table 3: Comparative data on PMGL and PGL (pectin lyase) enzyme production by *P. cyclopium* in free and immobilized culture

Cultivation Method	PMGL and PGL (pectin lyases),U·mL^{-1}	Cultivation duration, days							
		4	7	10	13	16	19	21	24
Batch, free-cell cultures	PMGL	12.9	0	-	-	-	-	-	-
	PGL	15.9	0	-	-	-	-	-	-
Semi-continuous, immobilized cultures	PMGL	15.8	22.0	24.0	23.5	20.5	21.5	23.0	26.0
	PGL	16.0	23.1	25.0	24.5	22.2	22.5	26.0	28.5

Table 4: Advantages of cultivating *P. cyclopium* in an immobilized state compared to batch cultivation of free cells

No	Cultivation Parameters	Free Cells	Immobilized Cells
1	2	3	4
1	Cultivation duration (days)	4	40 and more
2	Volume of culture fluid obtained (mL)	85-90	900-1000
3	Number of passages required to obtain the same amount of culture fluid	13	1
4	Time of active pectin lyase production (day of cultivation)	4	7-13
5	Duration of stationary enzyme production phase (days)	1	6-7
6	Frequency of target product collection (interval in days)	4	3-4
7	Culture fluid activity		
	PMGL,U·mL^{-1}	12.9	26.0
	PGL,U·mL^{-1}	15.9	28.5
8	Need for culture fluid filtration	Required	Not required
9	Time and resource savings in inoculum preparation and equipment sterilization (fold)	-	12

Table 5: PMGL and PGL (pectin lyase) activity of variants isolated from the immobilized *P. cyclopium* 2-11 population.

Colony Type	Duration of Immobilized Culture Growth	Identified Culture Variants	Pectin Lyase Activity,U·mL^{-1}			
			Day 4	Day 8	Day 4	Day 8
			PMGL	PGL	PMGL	PGL
1	2	3	4	5	6	7
I	Inoculum of the original culture	1-1	5.0	5.7	6.5	7.9
II		1-2	8.5	10.1	10.1	12.9
III		1-3	12.9	16.0	14.2	17.5
IV	At the beginning of immobilization (after 8 days)	2-1	9.6	10.1	10.3	11.5
V		2-2	9.1	10.0	9.6	12.0
VI		2-3	15.4	17.0	18.0	19.5
VII		2-4	5.0	5.5	5.9	6.0
VIII		2-5	5.2	6.0	5.9	7.5
IX		2-6	6.0	6.5	6.9	7.3
X		2-7	7.2	7.9	8.0	9.0
		2-8	6.5	7.0	7.5	8.0
XII	After prolonged cultivation (after 30 days)	2-9	7.5	8.0	8.5	9.0
XIII		2-10	15.3	19.3	18.9	21.0
XIV		2-11	17.0	20.0	28.6	34.1
XV		2-12	7.5	8.0	8.5	9.0
XVI		2-13	6.1	9.1	7.0	10.0
XVII		2-14	7.8	8.0	10.0	9.6
XVIII		2-15	5.2	6.0	5.8	6.6

better mass transfer, improved control and reproducibility, protection from shear forces, reduced protease activity, and reduced contamination risk. These advantages were attributed to the improved conditions for the metabolic activity of immobilized cell associations, where the mycelial hyphae grew linearly across the surface of the carrier without forming pellets (Carvalho et al., 2002). This arrangement ensured maximum accessibility of nutrients and oxygen to all cells. Adsorptive immobilization of *P. cyclopium* spores on the carrier resulted in the formation of a filamentous-sponge-like mycelial structure. Under continuous cultivation with periodic replacement of the nutrient medium, the immobilized mycelium was capable of producing pectin lyase enzymes continuously for over 40 days. Enzymatic activity in the culture filtrates increased from 12.9–15.9U·mL^{-1} to 26.0–28.5U·mL^{-1}, while overall culture productivity doubled, and volumetric efficiency increased significantly.

The stationary phase of growth and enzyme production was extended to 6–7 days. Enzymatic activity increased 1.8–2.0-fold, volumetric productivity grew tenfold, and filtrate collection could be performed more than twelve times, as compared to a single collection in batch cultivation. The number of passages required was reduced twelvefold. Filtration of the culture fluid was no longer necessary. Furthermore, the process significantly reduced the consumption of time, materials, and inoculum, demonstrating clear technological and economic benefits for the production of pectin lyases from *P. cyclopium* (Quong, 2002). Significant improvements in the biosynthetic activity of *P. cyclopium* were achieved through its immobilization. The immobilization of micromycetes on solid carriers simulates their natural growth environment and facilitates the formation of diverse morphological forms. It was found that immobilized cultivation of *P. cyclopium* led to enhanced enzymatic activity in the culture fluid, improved culture productivity, and prolonged periods of active enzyme biosynthesis. This was attributed to the more favorable biosynthesis conditions afforded by the immobilized filamentous-sponge-like structure formed on the carrier, compared to conventional batch cultivation. This aligns with the studies of Tan et al. (2021) and Costa et al. (2023) on the immobilized cultivation of *P. cyclopium*. Although their studies were on focused on a different biotechnological industry, increased efficiency and enhanced enzymatic activity were recorded.

Analysis of the inoculum revealed the presence of three colony types, designated as I, II, and III. Type I (1-1) exhibited a white margin, radial wrinkling, a slightly raised center, and blue-green aerial mycelium (Fig. 3 and 4). A clearly defined ring of lighter coloration appeared in the center, and the colony diameter was 4.7 cm. Type II (1-2) was morphologically similar but distinguished by the presence of yellow exudate and a slightly larger diameter of 5.0 cm. Type III (1-3) was smaller in size (3.8 cm), with a white margin and blue-green aerial mycelium, but lacked exudate. The pectin lyase activity in these variants ranged from 6.5 to 14.2U·mL^{-1} for PMGL and from 7.9 to 17.5U·mL^{-1} for PGL. To assess the variability of the immobilized culture, mycelial samples were taken after the second medium replacement (day 8) and again after extended cultivation (30–40 days). All samples were obtained by carefully removing mycelium from the surface of the immobilized structure. A total of 60 mycelial samples were analyzed. Among 30 early samples, eight colony types were identified and designated as types IV through XI. Each sample contained two or three predominant colony types. Type IV (2-1) exhibited a transparent margin, followed by a broad white ring, radial wrinkling, and a pale green spore zone, with a sterile raised center. The colony diameter was 3.5–3.6 cm. Type V (2-2) showed a white marginal ring and radial wrinkling, but its center was flat, surrounded by a spore zone, with a diameter of 3.5 cm. Type VI (2-3) had a similar margin but a grey-green spore zone and a flat center covered with a white coating. Its diameter reached 5.0 cm. Type VII (2-4) displayed a white central ring, a non-sporulating aerial mycelium, and a blue-green spore zone that gradually lightened toward the edges in a rainbow-like pattern. Its diameter was 7.7 cm. Type VIII (2-5) had a raised center with a white coating, a white marginal ring, and grey-blue aerial mycelium. The colony diameter was 7.6 cm. Type IX (2-6) had yellow exudate droplets and a flat center, with a diameter of 5.2 cm. Type X (2-7) had irregular margins and yellow exudate in the center, with a diameter of 4.5 cm. Type XI (2-8) showed radial wrinkling at the outer edge and blue-grey aerial mycelium, with a colony diameter of 5.6 cm (Fig. 11).

Mono-spore suspensions were prepared from each identified colony type. After plating on agar, isolated variants were cultivated in immobilized conditions, and pectin lyase production was measured in the culture fluid (Hu et al. 2003). Among the variants isolated after immobilization, variant No. 2-3 demonstrated the highest enzyme activity, being 3.0–3.2 times more active than other variants. Further analysis was performed after extended cultivation. On day 30, 30 additional mycelial samples were collected, and seven new colony types were identified and designated as types XII through XVIII. Type XII (2-9) showed delayed sporulation, with initial growth being aspore, followed by the appearance of pale green spores on day 6–7 (Fig. 12). It exhibited a white margin and a slightly raised sterile center. One-quarter of the colony remained aspore, while the remaining three-quarters were sporulating. The diameter was 6.3 cm. Type XIII (2-10) was entirely aspore, with radial wrinkles and a slightly raised, wrinkled center, with a diameter of 3.1 cm. Type XIV (2-11) was similarly asporic with radial folding and a flat white surface (Fig. 13), measuring 4.2–4.5 cm. Type XV (2-12) had a beige coating in the center, a raised center, radial folding, and a diameter of 3.5 cm. Type XVI (2-13) was white and fluffy with a beige center, flat and smooth, with a diameter of 2.1 cm. Type XVII (2-14) had irregular margins, a flat center, beige coating, and radial folding, with a diameter of 3.1 cm. Type XVIII (2-15) also had a beige coating, radial folding, and a flat center, with a diameter of 3.3 cm (Fig. 14). Mono-spore suspensions were again prepared from all these variants. Cultures were grown under immobilized conditions for 8 days, and pectin lyase activity was measured. After prolonged cultivation, several of the newly isolated variants outperformed variant 2-3 in enzyme productivity. Notably, variants No. 2-10 and No. 2-11 showed the highest activity, with variant No. 2-11 demonstrating a 5.0–5.2-fold increase in enzymatic activity over all other variants. Variant 2-11 dominated the population, comprising 87% of the colony types. As a result, it was designated as the new high-activity strain *P. cyclopium* 2-11.

The *P. cyclopium* 2-11 strain differed from the original culture in morphological characteristics. Colonies measured 4.2–4.5 cm in diameter, were aspore, exhibited radial wrinkling, and had a white, non-sporulating surface. In terms of pectin lyase production, the newly obtained strain surpassed the original culture by 6.2–6.6 times.

The study of population variability in the immobilized culture of *P. cyclopium* revealed that despite favorable growth conditions in the filamentous-sponge structure formed during immobilization, the fungal population on the carrier was subject to morphological changes. In response to immobilization, eight distinct colony types emerged during the early growth phase of *P. cyclopium*, identified across thirty samples (Fig. 5-10). After extended cultivation, an additional seven colony types appeared, also identified in thirty samples. In total, fifteen variants differing in structural and morphological traits and enzyme productivity were isolated. Among these, variant 2-11, obtained after prolonged cultivation of the immobilized culture, exhibited the highest activity. Thus, immobilization and long-term cultivation of *P. cyclopium* on a solid support led to population variability in both morphology and enzymatic productivity. As a result of this study, a highly active variant was selected – strain *P. cyclopium* 2-11 – which produced pectin lyase enzymes in concentrations of up to 28.6U·mL^{-1} and 34.1U·mL^{-1}. This activity exceeded that of the same culture after physiological optimization by a factor of 3.6–4.3 and surpassed the originally selected strain by 6.2–6.6 times. The observed population variability attributed to immobilization and long term cultivation disagrees with the works of Carvalho et al. (2022), who reported on their study on advances on bacterial and fungal biofilms for the production of added-value compounds that long term immobilization extended enzyme production with a 4.5 fold increase in productivity (Admanova et al., 2024). In addition, Genthon (2024) proposed that the cell type plays a critical role in determining the occurrence of diverse colonies during immobilization. More research is needed to further consolidate this occurrence.

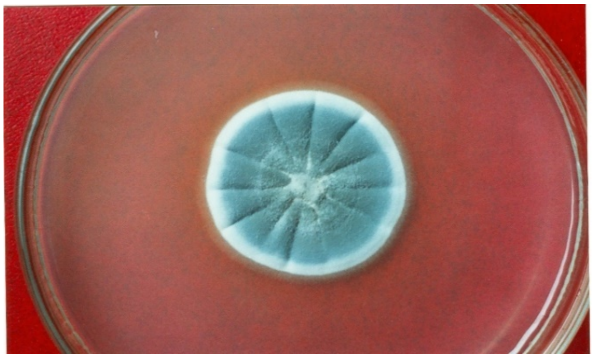


Fig. 3: Morphology and structure of variant I (1-1) identified in the inoculum of the original *P. cyclopium* culture. **Fig. 2 missing**

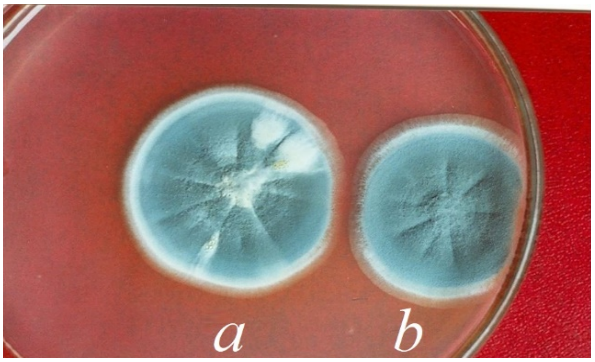


Fig. 4: a, b. Morphology and structure of variants II (1-2) and III (1-3) identified in the inoculum of the original *P. cyclopium* culture

Fig. 5: Identified colony type IV (2-1) after immobilization of *P. cyclopium*

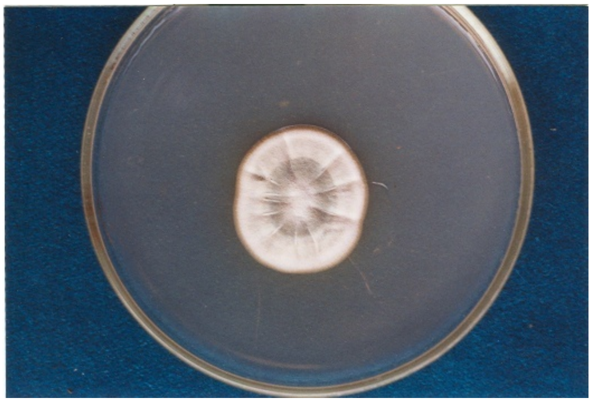


Fig. 6: Identified colony type V (2-2) after immobilization of *P. cyclopium*

Fig. 7: Identified colony type VI (2-3) after immobilization of *P. cyclopium*

Fig. 8: Identified colony type VII (2-4) after immobilization of *P. cyclopium*

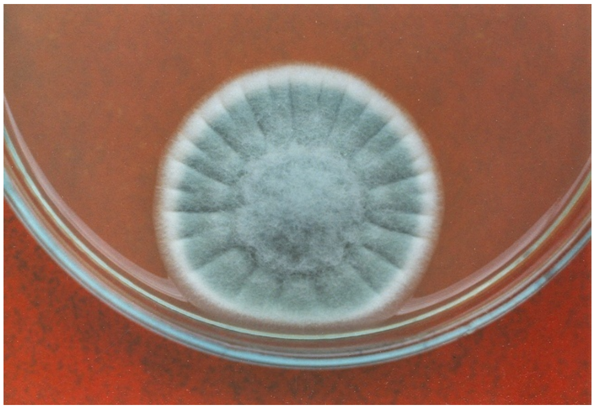


Fig. 9: Identified colony type VIII (2-5) after immobilization of *P. cyclopium*

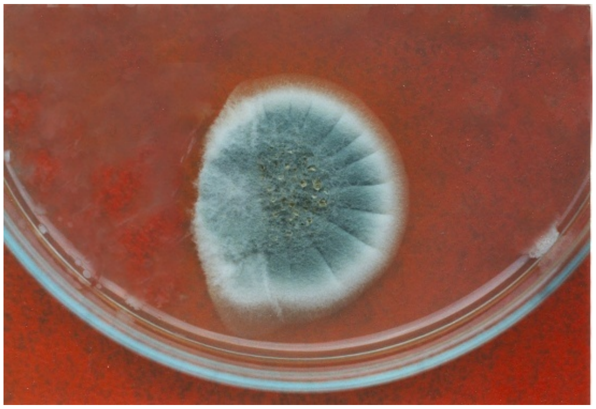


Fig. 10: Identified colony type IX (2-6) after immobilization of *P. cyclopium*

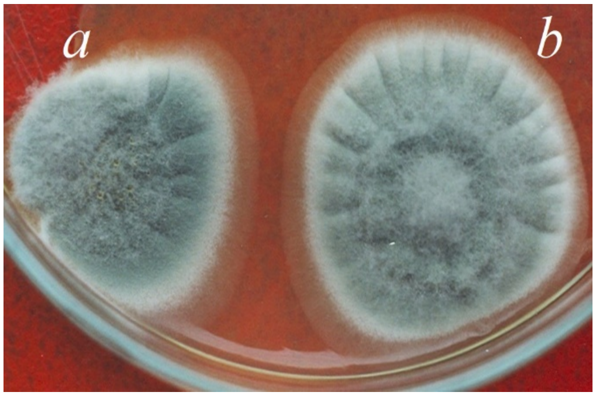


Fig. 11: a, b. Identified colony types X (2-7) and XI (2-8) after immobilization of *P. cyclopium*

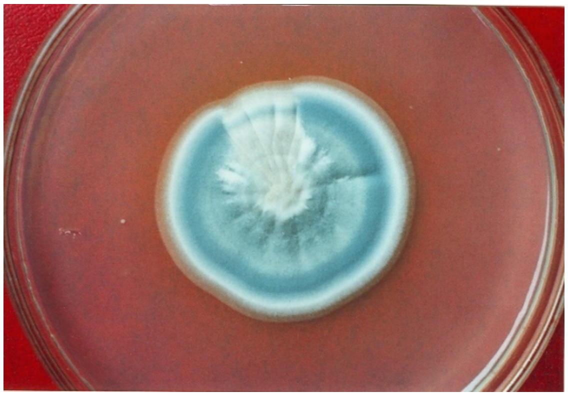


Fig. 12: Identified colony type XII (2-9) after prolonged cultivation of *P. cyclopium*

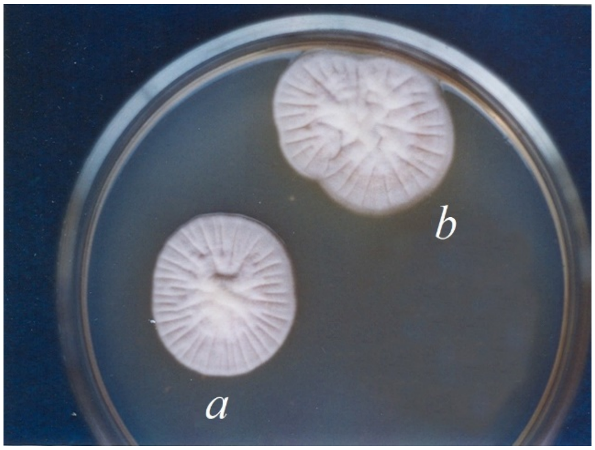


Fig. 13: a, b. Identified colony types XIII (2-10) and XIV (2-11) after prolonged cultivation of *P. Cyclopium*.

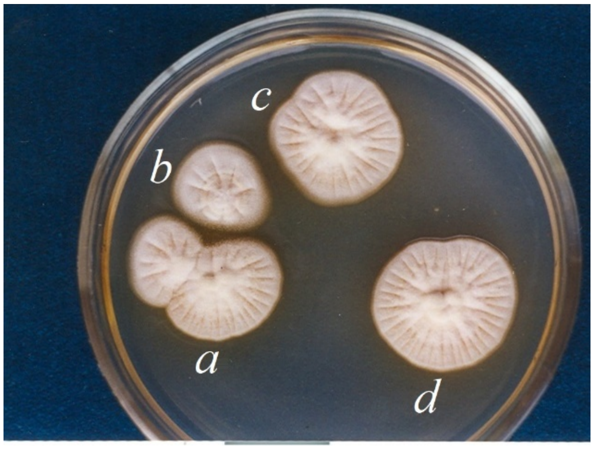


Fig. 14: a, b. Identified colony types XV (2-12), XVI (2-13), XVII (2-14), and XVIII (2-15) after prolonged cultivation of *P. cyclopium.*

Production of Pectin Lyase Enzymes from *P. cyclopium* 2-11, Their Physicochemical Properties, and Application in Synthetic Detergents

Pectolytic enzymes are widely used in the juice and wine industries for clarifying fruit, berry, and vegetable juices and for wine production. This application is one of the oldest uses of these enzymes and still accounts for their widespread commercial distribution. The action of pectin-degrading enzymes results in a rapid reduction in the viscosity of fruit and vegetable juices, decreased turbidity, and increased yield in terms of the dry matter of plant raw materials. One of the potential applications of pectolytic enzymes is the treatment of softwood species such as Sitka and Norwegian spruce, which are used in industry. When treated with pectolytic enzyme preparations or specialized bacteria that produce these enzymes, the resulting sapwood becomes more susceptible to microbial degradation and processing. Some studies demonstrated that storing wood in freshwater lakes in the presence of such bacteria was more effective in increasing permeability than treatment with chemical preservatives (Abdrashitova et al., 2002, Kyllönen et al., 2023).

There are also reports on the use of pectolytic enzymes in animal husbandry, particularly in feed formulations for various agricultural animals. These enzymes improve nutrient digestibility and, consequently, animal productivity. Positive outcomes were observed in feeding trials with poultry and young cattle. By the second month of feeding, physiological and biochemical studies showed that enzyme-supplemented beet pulp diets improved digestibility across all nutrient groups. Additionally, animals receiving enzymatic supplements exhibited weight gains up to 19% greater than control animals. Pectolytic enzymes also play an important role in the pharmaceutical industry. For instance, the use of pectoclostridin G10X during the extraction of diosgenin from the rhizomes of *Dioscorea caucasica* Lipsky increased yield by 16%.

Our research proposes a novel application for pectin-degrading enzymes—as active additives in laundry detergents to improve their efficacy against carbohydrate-based stains on textiles. The concept is based on the substrate specificity of pectin lyase enzymes, which are capable of breaking down complex pectic carbohydrates under alkaline conditions into simpler, water-soluble compounds. The idea of applying pectin lyase enzymes from *P. cyclopium* 2-11 in detergent formulations required the production of concentrated enzyme preparations with high pectin-degrading activity. In laboratory and industrial practice, organic solvents and neutral salts are commonly used for enzyme precipitation. Typical precipitants include

96.5% ethanol, 98.0% isopropanol, chemically pure acetone, and ammonium sulfate. When using ammonium sulfate, an additional dialysis step is needed to remove salts from the isolated protein.

The precipitation of proteins by organic solvents is based on reduced solvation of polar groups on the enzyme surface. Water molecules associated with hydrophobic regions of the protein can be displaced by solvent molecules, leading to reduced solubility and subsequent protein aggregation and precipitation. For isolating pectin lyase enzyme preparations from the culture fluid of the immobilized *P. cyclopium* 2-11, we employed ethanol as the traditional precipitant at a ratio of 1:4. The resulting yield of the ethanol-precipitated enzyme preparation was 2 grams per liter of culture fluid.

The ethanol-precipitated enzyme preparation, obtained at pH 10 and in a yield of 2 grams per liter, was biochemically analyzed for the presence of common enzymes. The biochemical analysis showed that the enzyme preparation was heterogeneous. In addition to pectin lyases, it contained polygalacturonase and protease as accompanying proteins (Table 6). Among the most important characteristics of enzyme preparations that determine their practical applicability are pH optimum, temperature optimum, and substrate specificity. Our findings indicate that changes in hydrogen ion concentration influence the pectin lyase activity of the preparation, with activity increasing under mildly alkaline conditions and decreasing at acidic and neutral pH values (Fig. 15). It was established that the optimal pH range for the activity of pectin lyase enzymes produced by *P. cyclopium* 2-11 lies between 10 and 11. This optimum (pH 10–11) is consistent with, but slightly higher than, the general range (pH 8–10) reported for fungal pectin lyases, reflecting the enzyme's strong alkaline adaptation.

Table 6: Characteristics of the ethanol-precipitated enzyme preparation obtained from *P. cyclopium* 2-11

No	Enzyme Type	Activity Value, U/g
1	Pectin lyase (PL)	143.3
2	Polygalacturonase	5.3
3	Protease	3.5
4	α-Amylase	-

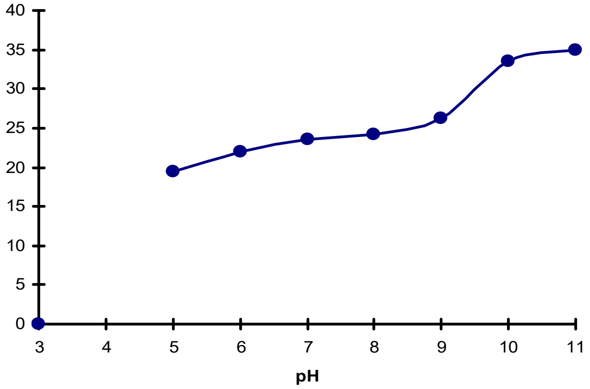


Fig. 15: Determination of the pH optimum for the enzymatic activity of the preparation obtained from *P. cyclopium* 2-11.

The enzymes under study exhibit activity only within a specific pH range. Therefore, to assess the effect of temperature on the stability of *P. cyclopium* 2-11 pectin lyases, experiments were conducted at pH 10 for one hour under varying temperature conditions. It was found that the enzymatic reactions exhibited a bell-shaped dependence of enzymatic activity on temperature, with peak activity observed at 50–60°C (Fig. 16). Increasing the temperature to 70°C resulted in a sharp decline in pectin lyase activity. In the study of pectin lyase enzymes, determining their substrate specificity is of great significance. The available literature lacks data on the substrate specificity of pectin lyases produced by *Penicillium* species. Therefore, we investigated the ability of these enzymes to hydrolyze glycosidic bonds in various pectic substances. Pectin lyases are highly substrate-specific enzymes that catalyze precisely defined biochemical reactions without generating undesirable by-products. They specifically cleave the α-1,4-glycosidic linkages in highly esterified pectic substances through a β-elimination mechanism, resulting in the formation of unsaturated oligogalacturonides as the primary reaction products.

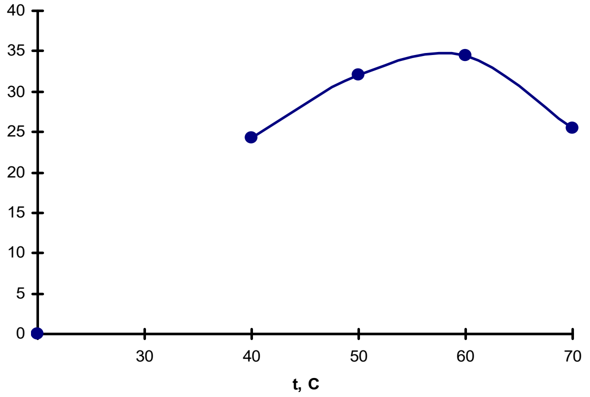


Fig. 16: Thermostability of the enzyme preparation obtained from *P. cyclopium* 2-11.

As substrates, we used rhamnogalacturonans and pectic acid with varying degrees of esterification. At the same enzyme concentration, the least esterified D-galacturonan (78%) was most actively degraded by polygalacturonate lyase. This was followed by highly esterified D-galacturonan (96%), which was degraded by polymethylgalacturonate lyase. Pectic acid was the least susceptible to hydrolysis. For the practical application of the enzyme preparation, it is important to assess the effect of a highly alkaline detergent environment on its stability under prolonged exposure. We studied the influence of a high pH environment (pH 12) typical of synthetic detergents and the duration of exposure (1 hour) on the activity of *P. cyclopium* 2-11 pectin lyases at 20–60°C. The results showed that prolonged exposure to alkaline detergent conditions does not affect the stability of *P. cyclopium* 2-11 pectin lyase enzymes, even at temperatures of 50–60°C

Thus, the pH and temperature optima, as well as the substrate specificity of the studied enzymes, were established. The enzyme preparation demonstrated the highest stability at temperatures of 50–60°C, with a pH activity optimum in the range of 10–11. Substrate specificity was confirmed, with both low- and high-esterified D-galacturonans identified as optimal substrates. Furthermore, exposure to highly alkaline detergent conditions, even at 50–60°C, did not affect the stability of the pectin lyase enzymes. Numerous species of fungi were found to be capable of breaking down pectin during the first screening of fungal cultures. Only a small number, nevertheless, were able to function effectively in the alkaline environment needed for pectin lyase activity. With enzyme production noticeably higher than the parent culture, the recently isolated *P. cyclopium* 2-11 strain proved to be a high-performing strain. Because it can break down both highly esterified and low-esterified D-galacturonan at pH values between 10 and 11, this strain is well suited for industrial settings where these kinds of conditions are common, like detergent formulation.

The idea of applying pectin lyase enzymes in laundry detergents emerged after the determination of their optimal pH and temperature activity profiles. The effectiveness of *P. cyclopium* 2-11 pectin lyases in removing carbohydrate-based stains when incorporated into detergent formulations was tested both under laboratory conditions and in an industrial setting.

In laboratory experiments, white cotton fabric was used, onto which carbohydrate-rich stains (from berries, fruits, vegetables, and wine) measuring 3.0–3.5 cm in diameter were applied. After 24 hours, the stained areas were treated under different washing conditions: Variant 1 – synthetic detergent (control); Variant 2 – synthetic detergent with 10mL of enzyme added; Variant 3 – household soap; Variant 4 – household soap with 10mL of enzyme added (Table 7).

As a result of these experiments, it was established that neither synthetic detergents nor household soap alone were capable of completely removing the stains. Only the combined use of the enzyme preparation with synthetic detergent produced a synergistic effect in stain removal (En-Hendawy et al., 2002; Khazi et al., 2025). Under industrial conditions, the test materials included table linens and kitchen staff uniforms from a cafeteria, all containing various types of stains. The control group consisted of items washed using a standard synthetic detergent without enzymes for 1 hour at a temperature of 50–60°C. In the first experimental variant, the laundry was washed in a standard cycle with the addition of the enzyme preparation. In the second variant, the same procedure was followed, but the amount of detergent used was reduced by half compared to the control (Table 8). It is impossible to overestimate the economic impact of producing enzymes using immobilized *P. cyclopium* 2-11 (Maghraby et al., 2023). In addition to increasing enzyme productivity, the immobilization technique employed in this study also decreased labor and material expenses. Significant time and resource savings are indicated by the 12-fold decrease in inoculum consumption and the removal of the requirement for culture fluid filtration, which may result in lower operating costs for the production of enzymes on a large scale. Using pectin lyase enzymes in detergent formulations is an environmentally friendly strategy. Conventional detergents frequently use harsh chemical agents to remove stains, which may be harmful to the environment. The detergent industry can lessen its dependency on artificial chemicals by using enzymes that are derived from biological sources (Tanwar et al., 2021).

The conducted experiments demonstrated the positive effect of pectin lyase enzymes on enhancing the efficiency of detergents. While synthetic detergents alone were not capable of removing carbohydrate-based, pectin-containing stains, their combination with the enzyme preparation produced a synergistic effect, resulting in complete stain removal with water. Washing quality increased to 90%—the fabric appeared clean and white, with only minor beige discolorations remaining. These residual stains were likely starch-based carbohydrates that remained intact due to the absence of α-amylase in the enzyme preparation. It was established that with a detergent dosage of 10g per 1kg of laundry and the addition of 0.5% pectin lyase enzymes (relative to the detergent weight), washing performance increased from 65% to 90%, representing a 25% improvement. Moreover, incorporating 0.5% pectin lyase enzymes into laundry formulations enabled a twofold reduction in detergent consumption. Although, enzyme stability under alkaline conditions were tested, the shelf life of the enzymes in real-world detergent formulations over extended periods remain uncertain and future research is recommended to understand this.

Table 7: Treatment of pectin-containing stains with pectin lyase enzymes from *P. cyclopium* 2-11

No	Experimental Variant	Pectin-containing stains from												
		apple	peach	raspberry	blackberry	apricot	sweet cherry	red wine	plum	grape	sour cherry	banana	carrot juice	beet juice
1	Detergent (control)	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX
2	Detergent + enzyme	X	O	O	O	X	X	O	X	O	X	X	X	X
3	Household soap	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX
4	Household soap + enzyme	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX

Legend: XXX – control (stains not removed with water); XX – weak stain removal effect; X – good stain removal effect; O – complete stain removal

Table 8: Use of pectin lyase enzymes to enhance the effectiveness of synthetic detergents

No.	Experimental Variant	Amount of Detergent (g/kg of laundry)	Amount of PL Enzyme Added to Detergent (%)	Efficiency (%) of Detergent and PL Enzyme Action
1	Detergent only (control)	10	-	65
2	Detergent + enzyme preparation	10	0.5	90%
3	½ Detergent + enzyme preparation	5	0.5	90%

Notwithstanding the encouraging outcomes, this study has some limitations. Protease and polygalacturonase were among the accompanying activities in the heterogeneous enzyme preparation that was produced by ethanol precipitation. Although the primary pectin lyase activity was strong, the presence of these extra enzymes may have unforeseen consequences in some situations, such as possible harm to textiles' protein-based fibers if they are used in detergents over an extended period of time. Earlier research that described physical limitations under NMR spectroscopy and diffusion limitations when heterogeneous precipitation was involved also supports this limitation (De Man et al., 2021; Li et al., 2021). Furthermore, little is known about the genetic and molecular underpinnings of the *P. cyclopium* 2-11 strain's remarkable alkaline stability and observed hyper-productivity. It would be crucial to comprehend the underlying mutations or regulatory alterations that led to its improved phenotype in order to improve strains further and gain an elementary grasp of fungal enzymology. Furthermore, although the enzymes' operational stability under high-temperature, alkaline conditions was shown, the enzyme preparation's stability and long-term shelf life in a commercial solid or liquid detergent formulation were not assessed.

Therefore, in order to determine the distinct kinetic parameters and functional roles of each pectin lyase isoform, it is recommended that future research should concentrate on their purification and characterization. It is also strongly advised to employ omics technologies to clarify the genetic factors underlying the strain's superior characteristics. To move this promising biocatalyst from the lab to the industrial setting, thorough research on how to formulate the enzyme into a stable, commercially viable detergent product, including tests conducted over long storage times under various conditions, is crucial. The developed pectin-lyase preparation is intended for potential use as a detergent additive; therefore, its handling and application were considered from a safety and regulatory perspective. The enzyme solution is non-toxic and biodegradable, but, like all protein products, it may cause irritation or sensitization in susceptible individuals. Laboratory handling followed standard occupational hygiene for enzyme preparations, including the use of gloves, protective eyewear, and closed containers to minimize aerosol formation. Preliminary storage tests showed that the lyophilized or ethanol-precipitated preparation retained more than 85% of its initial activity after three months at 4 °C, indicating satisfactory short-term stability. Compatibility tests during formulation demonstrated that the enzyme remained active in the presence of non-ionic surfactants and moderate levels of bleaching agents, suggesting practical applicability in commercial detergent matrices. All liquid residues were inactivated by heating to 80 °C for 30 min before disposal to ensure safe environmental release. Further studies on long-term shelf-life, allergenicity assessment, and regulatory certification will be performed prior to any industrial or consumer-product deployment.

Conclusion

This study presents a comprehensive approach to optimizing the biosynthesis, characterization, and practical application of pectin lyase (PL) enzymes produced by *P. cyclopium*. Through the selection of an efficient microbial producer, optimization of cultivation conditions, and assessment of enzymatic properties, a novel strategy was developed to enhance the effectiveness of enzymatic additives in detergent formulations. Optimal sources of carbon and nitrogen for the cultivation of *P. cyclopium* were identified. Fructose was shown to be the most effective carbon source for pectin lyase production, while ammonium chloride was the preferred nitrogen source. The biosynthesis of the studied enzymes was found to be constitutive in nature. As a result of investigating the population variability of the immobilized *P. cyclopium* culture, a highly active strain – *P. cyclopium* 2-11 – was isolated. This strain demonstrated significant differences from the parent culture in terms of morphology, physiology, and enzyme production, exceeding the original strain in pectin lyase activity (PMGL and PGL) by 6.2–6.6 times.

An ethanol-precipitated enzyme preparation was obtained from the culture fluid of the immobilized strain, and its physicochemical properties were studied. The ethanol-precipitated enzyme preparation showed optimal activity at pH 10–11 and 50–60°C, retained stability under alkaline conditions, and effectively degraded both high- and low-esterified D-galacturonans. Laboratory and pilot-scale washing trials confirmed that the inclusion of the enzyme preparation increased detergent performance by approximately 25% and allowed a twofold reduction in detergent dosage.

While these results confirm the laboratory-scale feasibility of producing and applying alkaline pectin lyases from *P. cyclopium* 2-11, their long-term storage stability, compatibility with commercial detergent formulations, and large-scale economic performance require further validation. Therefore, the strain can be regarded as a promising source of alkaline pectin lyases for sustainable and potentially cost-effective detergent biotechnology, pending industrial formulation and regulatory testing.

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