Cell wall disruption by microbial enzymes from soil isolates for efficient downstream processing of *Rhodotorula toruloides* for lipid production

Functional Screening and Genomic Characterization of Soil Microbes with Yeast-Lytic Capabilities

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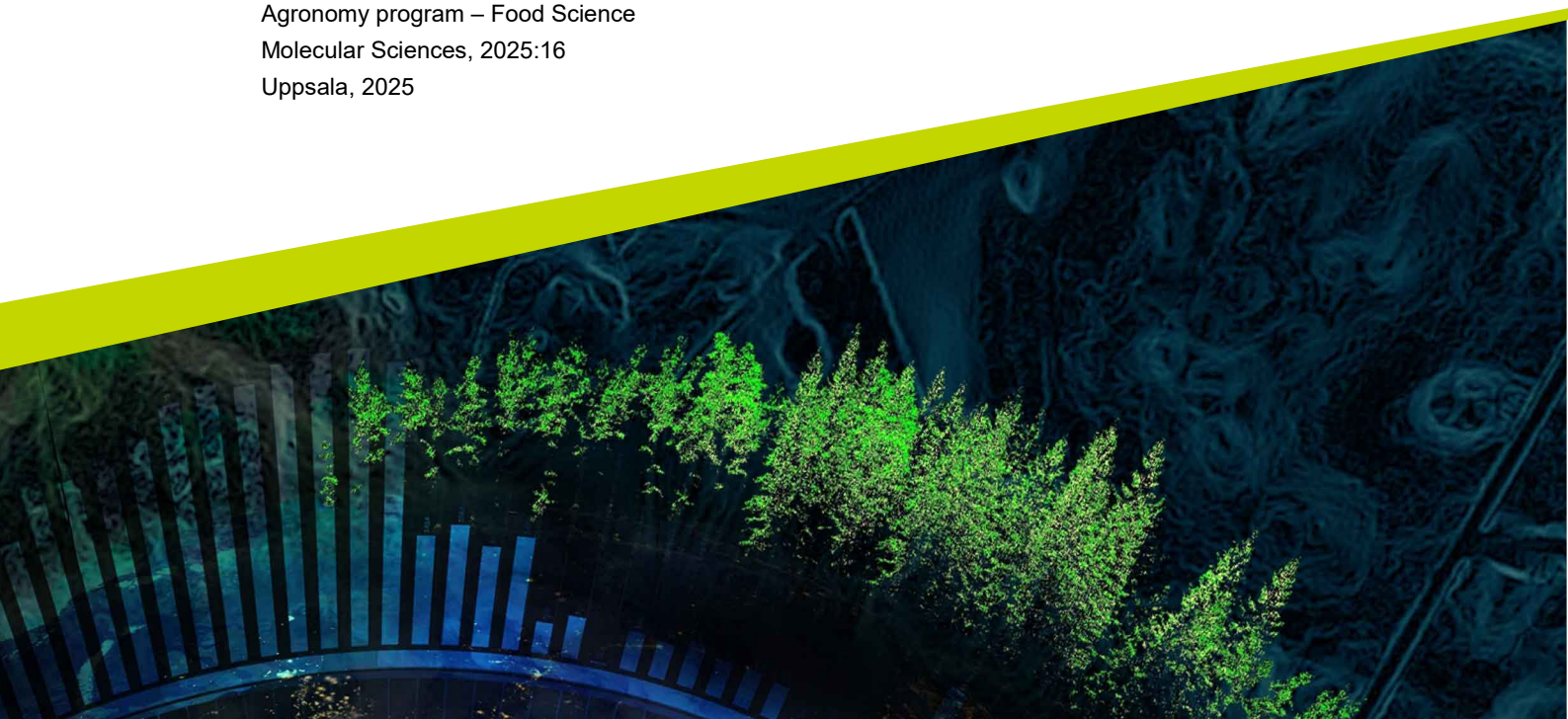

Cell wall disruption by microbial enzymes from soil isolates for efficient downstream processing of *Rhodotorula toruloides* for lipid production.

Functional Screening and Genomic Characterization of Soil Microbes with Yeast-Lytic Capabilities

Jäst cellväggsnedbrytning av mikrobiella enzymer från jordisolat för effektiv nedströmsbearbetning av Rhodotorula toruloides för lipidproduktion

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Abstract

Rhodotorula toruloides *is an oleaginous yeast recognized for its high lipid content, high production of oils and carotenoids and favorable fatty acid profile, making it a valuable candidate for food, feed, and biofuel applications. However, the robust structure of its cell wall poses a major challenge for cost-effective lipid extraction. This study aimed to identify soil-derived microbial strains capable of enzymatically degrading* R. toruloides *cell walls to improve biomass accessibility and processing efficiency.*

Ten microbial isolates, including bacteria, filamentous fungi, and yeasts were cultivated in media containing R. toruloides *cell wall material as the sole carbon source. Growth was assessed via optical density measurements (OD_{600}), results were further validated and assessed through biomass quantification using dry cell weight (DCW) analysis. The two methods produced consistent trends, reinforcing the observed differences in growth performance.*

Among the isolates, Streptomyces seoulensis *(strain 5) demonstrated the highest growth across both OD_{600} and DCW measurements. 16S rRNA gene sequencing confirmed its identity with >99% sequence similarity to* S. seoulensis. *Genome annotation revealed a broad spectrum of cell wall-degrading enzymes, including multiple glucanases, chitinases, proteases, and GPI-cleaving enzymes, but no mannan-degrading enzymes were detected.*

These findings suggest that S. seoulensis *is a promising candidate for enzymatic pretreatment of* R. toruloides*, supporting more sustainable lipid extraction methods in biotechnological applications.*

Keywords*:* Rhodotorula toruloides, Streptomyces seoulensis*, enzymatic degradation, optical density, biomass quantification, dry cell weight, microbial screening, lipid extraction and protoplasts*

Contents

Abbreviations

Abbreviation	Full Term
ANI	Average Nucleotide Identity
BLAST	Basic Local Alignment Search Tool
CAZy	Carbohydrate-Active enzymes database
CDS	Coding DNA Sequence
DCW	Dry Cell Weight
DNA	Deoxyribonucleic Acid
EC Number	Enzyme Commission Number
FAA	FASTA Amino Acid file
FNA	FASTA Nucleotide file
GFF	General Feature Format
GH	Glycoside Hydrolase
GPI	Glycosylphosphatidylinositol
GTDB	Genome Taxonomy Database
NCBI	National Center for Biotechnology Information
OD_{600}	Optical Density at 600 nm
PCR	Polymerase Chain Reaction
R. toruloides	Rhodotorula toruloides
RNA	Ribonucleic Acid
S. seoulensis	Streptomyces seoulensis
rRNA	Ribosomal Ribonucleic Acid
tRNA	Transfer Ribonucleic Acid

Introduction”

Background and Importance

Microbial lipid production has gained significant attention as a sustainable alternative to plant-based oils. The oleaginous yeast *Rhodotorula toruloides* is a promising candidate for single-cell oil (SCO) production, with a lipid profile similar to rapeseed oil, making it suitable for food, feed, and biodiesel applications (Wu et al., 2023). *R. toruloides* is a non-conventional, red basidiomycetous yeast with notable biotechnological potential due to its ability to accumulate lipids constituting up to 70% of its dry cell weight, primarily in the form of triacylglycerols. These lipids closely resemble plant oils, making them attractive feedstocks for biofuel, oleochemical production, food and feed material (Zhao et al., 2021).

Beyond lipid productivity, *R. toruloides* exhibits robust growth under diverse conditions, can metabolize a wide variety of carbon sources—including glucose, xylose, glycerol, and lignocellulosic hydrolysates—and is tolerant to inhibitory compounds such as furfural and acetic acid (Xue et al., 2024). It also naturally synthesizes carotenoids like β-carotene and torularhodin, which are valued in the

food, pharmaceutical, and cosmetic industries (Ribeiro et al., 2023). Genomically, it possesses a high-GC content genome of approximately 20.5 Mb with over 9,000 genes (Martín-Hernández et al., 2021).
Recent advances have expanded the genetic engineering toolbox for *R. toruloides*, particularly with the development of the RT-EZ toolkit, which enables modular and efficient expression cassette assembly and integration. This system enhances capabilities for gene overexpression, metabolic pathway engineering, and functional genomic studies, all crucial for optimizing lipid yield and stress resistance (Koh et al., 2025).
Given its metabolic flexibility, natural lipid accumulation capacity, and improved genetic tractability, *R. toruloides* is increasingly seen as a sustainable host for lipid bioproduction. However, its rigid cell wall structure presents a major obstacle to efficient lipid extraction, necessitating effective cell wall disruption strategies (Jin et al., 2012).

Key Challenge

- The rigid cell wall of *R. toruloides* significantly hinders lipid recovery.
- Efficient cell wall degradation can markedly enhance lipid extraction yields (Kot et al., 2020).

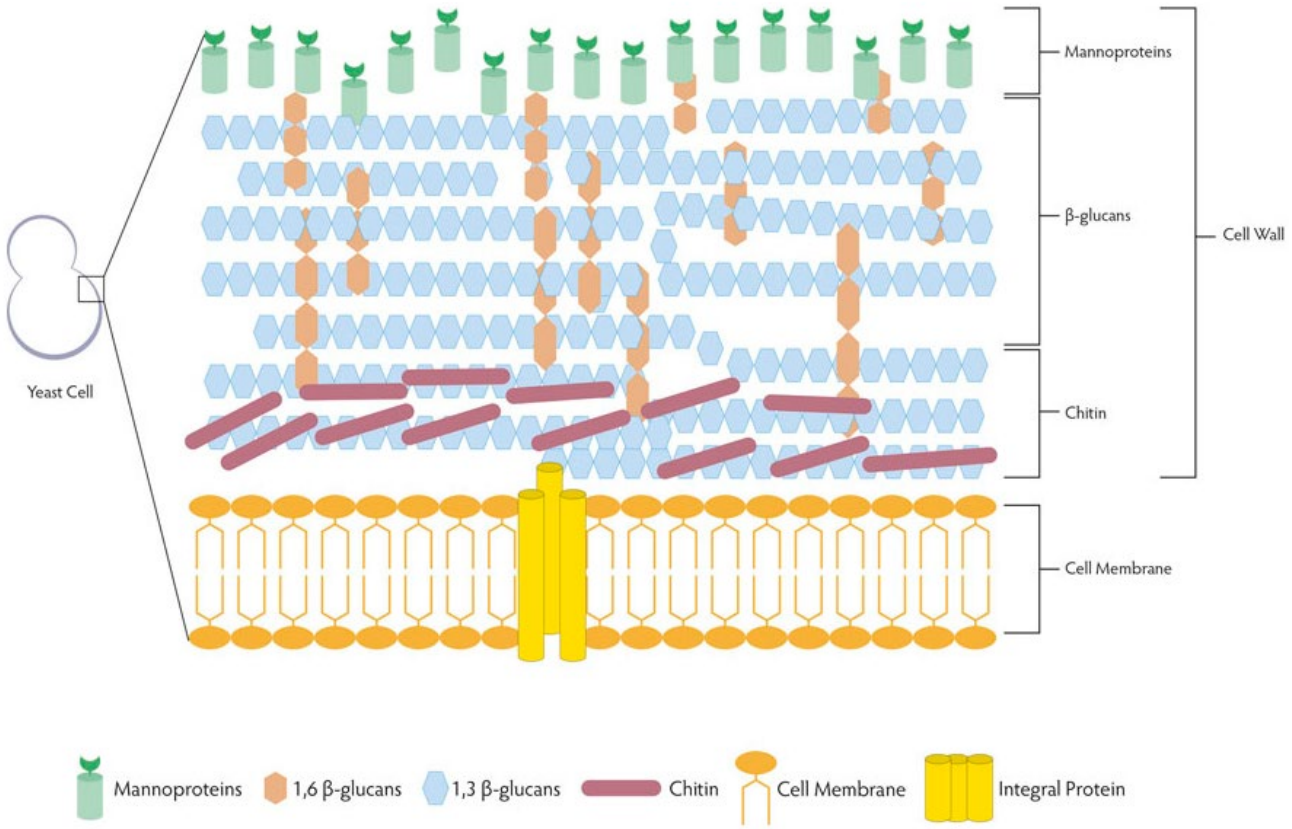

Figure 1. Schematic organization of the yeast cell wall, illustrating its two primary layers: an inner layer rich in chitin and β-glucans, and an outer layer containing mannoproteins (Mazzoleni S.p.A., no date).

The yeast cell wall consists of two primary layers (figure 1):

- **Inner Layer**: Composed mainly of chitin, β-1,3-glucan, and β-1,6-glucan, which confer structural integrity (figure 1).
- **Outer Layer**: Contains mannoproteins, including GPI-anchored and Pir proteins, attached via ester and disulfide bonds to β-1,3-glucan. Non-covalently bound glycoproteins are also present.
- **Plasma Membrane**: Lies beneath the wall, regulating nutrient exchange, signaling, and maintaining interactions with the wall structure (Andreu & del Olmo, 2018). However the yeast cell wall of *Rhodotorula toruloides* may look completely different and other enzymes may be needed to disrupt and degrade it.

Potential Solutions

- Enzyme-assisted hydrolysis is an effective approach for disrupting yeast cell walls and improving lipid recovery (Jin, et al., 2012).
- Soil-derived microbial isolates—including bacteria and filamentous fungi—may naturally produce cell wall-degrading enzymes, offering a sustainable and cost-effective strategy (Zhang et al., 2017)
- Enzymes such as β-glucanases, chitinases, mannanases, and proteases play central roles in breaking down yeast cell wall components (Adams, 2004).
- Combining enzymatic hydrolysis with acid treatment or supercritical CO_2 extraction can further increase lipid yields (Hegel et al., 2011) and (Kruger et al., 2018).
- Commercial enzymes like zymolyase or custom enzyme cocktails can also be utilized for efficient cell disruption (Brady et al., 1994).

Research Objectives

1. The strategic goal of this study is to develop an efficient and cost-effective method for disrupting the robust cell wall of *Rhodotorula toruloides* to enable lipid extraction. The study focuses on the identification and characterization of enzymes produced by soil-derived microorganisms capable of degrading *R. toruloides* cell walls. Specific aims include:
2. **Isolate and identify soil microorganisms** that exhibit yeast cell wall-degrading activity.
3. **Confirm microbial identities** using PCR amplification, sequencing, and BLASTn analysis.
4. **Evaluate microbial growth** on media containing *R. toruloides* cell wall as the sole carbon source.
5. **Conduct literature studies** on relevant topics including lipid production by *R. toruloides*, enzyme activity assays, and molecular biology techniques used in microbial enzyme screening.

Significance of the Study

1. Sustainability & Cost Efficiency

- Traditional mechanical and chemical cell disruption methods (e.g., bead mills, high-pressure homogenizers) are energy-intensive and can damage sensitive molecules (Andrews & Asenjo, 1987).
- Enzyme-based methods provide biologically specific, low-energy alternatives that are more environmentally friendly (Chen et al., 2018).

2. Industrial Applications

- **Food & Feed**: Lipids similar to rapeseed oil can serve nutritional purposes (Wang et al., 2022) and (Salejda et al., 2020).
  Biodiesel: Research on oleaginous yeasts, including *Lipomyces starkeyi*, has demonstrated their potential for sustainable biodiesel production based on system analyses and greenhouse gas evaluations (Karlsson et al., 2016; Karlsson et al., 2017).

3. Microbial Biotechnology

- Enhances understanding of soil microbial diversity and their enzymatic mechanisms for yeast degradation.

Research Hypotheses

1. Certain soil microorganisms produce enzymes that degrade *R. toruloides* cell walls.
2. Microbial isolates with higher enzyme activity grow faster and degrade yeast more efficiently.

Research Aim and Questions

The aim of this thesis was to identify and characterize bacterial strains capable of degrading the cell walls of the oleaginous yeast *Rhodotorula toruloides*, with the goal of contributing knowledge that could support future improvements in lipid recovery processes.

This study addressed the following research questions:

1. Which bacterial strains from the SLU Food Science collection are capable of degrading *R. toruloides* yeast cell walls?
2. What is the growth performance of these strains when cultured on *R. toruloides* cell wall fractions?
3. What genes and enzymes are involved in yeast cell wall degradation can be identified in the genomes of these strains?

Expected Outcomes

1. Identification of microbial strains with potent cell wall-degrading activity.

Scientific Foundations & References

- *R. toruloides* cell wall composition: (Lee et al., 1981)
- Enzymatic extraction strategies: (Jin et al., 2012)
- Growth analysis methodology: (Madigan et al., 2020)

2 Method

2.1 Growth studies of microorganisms in liquid culture using *Rhodotorula toruloides* cell wall as sole carbon source

YPD Agar Plate Preparation

Agar plates were prepared using manually formulated yeast peptone dextrose (YPD) medium. The YPD medium was composed of 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract, with 15 g/L agar added as a solidifying agent. All components were dissolved in distilled water to the desired volume and sterilized by autoclaving at 121 °C for 15–20 minutes. After sterilization, the molten medium was poured into sterile Petri dishes (approximately 20 mL per plate) under aseptic conditions and allowed to solidify at room temperature.

Preparation of Nitrogen-Limited Liquid Medium

A nitrogen-limited liquid medium was prepared to support microbial growth using *R. toruloides* cell wall material as the primary carbon source. The medium contained the following components per liter:

- **Yeast Cell Wall Fraction**: 1 g/L
- **Yeast Nitrogen Base (YNB, without amino acids and with ammonium sulfate)**: 0.67 g/L
- **Deionized Water**: up to 1 L

All components were thoroughly mixed and the medium was sterilized by autoclaving at 121 °C before use. The medium composition was a modified version of that described by Shibasaki et al. (2008), who studied the isolation of bacteria producing yeast cell wall-lytic enzymes.

Preparation of Cell Wall Media (400 mL Total Volume)

To prepare 400 mL of *Rhodotorula toruloides* cell wall media, the following components were mixed:

- **4 mL** of disrupted *R. toruloides* CBS14 cell pellet
- **25 mL** of deionized water (used to resuspend the cell pellet)
- **40 mL** of Yeast Nitrogen Base (YNB) without amino acids (10× stock solution)
- **345 mL** of distilled water

The final mixture was stored at room temperature until use.

2.2 Extraction of Yeast Cell Walls from CBS14 *Rhodotorula toruloides* incubated in Yeast peptone dextrose (YPD) CultureThe method for acquiring cell wall material was inspired by Shibasaki et al. (2008), with the key difference that a French press cell disruptor was used to break open the cells.

Preparation of *Rhodotorula toruloides* Cell Walls

1. YPD Broth Preparation (800 mL Total Volume)
The yeast growth medium was prepared as follows:

- Yeast extract: 8 g
- Peptone: 16 g
- Dextrose (glucose): 16 g
- Distilled water: Up to 800 mL

All components were dissolved thoroughly, and the solution was autoclaved at 121 °C for 15 minutes, then cooled to room temperature.

2. Cultivation of *R. toruloides*
Two fresh colonies of *R. toruloides* CBS14 were inoculated into 300 mL of sterile YPD broth in a 500 mL Erlenmeyer flask. The culture was incubated at 25 °C with shaking at 150 rpm for 4–5 days.

Harvesting and Resuspension of Cell Biomass
Following cultivation, 300 mL of the *R. toruloides* culture was transferred into six 50 mL Falcon tubes. The tubes were balanced and centrifuged at 7,000 × g for 5 minutes at 22 °C to collect the yeast biomass. The supernatant was discarded, and the pellets were washed once with sterile deionized water to remove residual media. After washing, each pellet was resuspended in 30 mL of sterile water and prepared for further processing (e.g., cell disruption).

Cell Disruption and Isolation of Yeast Cell Walls
Yeast cell pellets were resuspended in a minimal volume of sterile deionized water and vortexed thoroughly in 50 mL Falcon tubes to ensure uniform suspension. Prior to use, the French press was rinsed with 70% ethanol followed by sterile distilled water to prevent contamination. The resuspended yeast suspension was then processed through the French press at **20,000 PSI** with **two passes** to ensure efficient cell lysis.
The resulting lysate was distributed evenly across multiple 50 mL Falcon tubes and balanced for centrifugation. Samples were centrifuged at **7,000 × g for 5 minutes at 22 °C**. Following centrifugation, the **pellet containing yeast cell wall material** was retained, while the **supernatant containing cytoplasmic contents** was discarded.
To purify the cell wall fraction, the pellet was washed once with sterile deionized water. The final purified yeast cell wall preparations were either **stored at 4 °C for short-term use** or **frozen at −20 °C for long-term storage**.

Microbial Inoculation

Agar Plate Inoculation

- Strains belonging to the species *Penicillium verruculosum*, *Cyphellophora olivacea*, and 11 other microbial species were streaked or spot-inoculated

onto prepared YPD agar plates. All strains were obtained from the strain collection of the Department of Molecular Sciences at SLU.
- Plates were incubated at 25–30°C for 3–5 days, and later kept at 4°C.

2.3 Liquid Culture Inoculation

Pre-cultures of each strain were prepared in circa 20 mL of liquid cell wall media. Pre-cultures were incubated overnight at 25°C and 150RPM. The following day, OD_{600} of the pre-culture was measured. 1 mL of pre-culture was transferred to a cuvette, and OD_{600} was measured using a spectrophotometer to ensure that all cultures started at the same initial concentration. Based on the measured OD_{600}, the required volume of pre-culture and cell wall media for inoculation was calculated to achieve the same initial starting concentration. Calculated using this formula: $C_1V_1=C_2V_2$,

C_1 = OD_{600} of preculture,

V_1= Volume needed of preculture for inoculation,

C_2 = Target OD_{600} of growth culture,

V_2 = Final volume of growth culture.

Example 1: If pre-culture OD_{600} = **1.0** and you need OD_{600} = **0.1** in **50 mL flasks**,
- **C_1 = 1.0**, **V1 = ?**, **C2 = 0.1**, **V_2 = 50 mL**
- Solve for **V_1** → $V_1 = (C_2 \times V_2) / C_1$
- V_1 = (0,1 x 50) /1
- V_1 = 5 ml
- Calculate how much of the total volume should be preculture and cell wall media by subtracting V_2 with V_1 example follows:
- $V_2 - V_1$ = Volume of cell wall media that needs to be inoculated
- 50 – 5 = 45 ml
- 5 ml of preculture and 45 ml of cell wall media are needed in this case.

- Liquid cultures were prepared by inoculating strain specific volumes of pre-culture and cell wall media determined from OD_{600} values of pre-cultures from each strain.
- Cultures were incubated at 25°C, shaking at 150 RPM to ensure aeration and proper mixing.

Liquid Culture Optical Density (OD_{600}) Measurements

- Growth was monitored by measuring optical density (OD_{600}) using a spectrophotometer.
- Four OD_{600} measurements were taken over a 7-day period for each strain.

- For each measurement, a 1 mL aliquot was collected from the liquid culture, transferred to a cuvette, and OD_{600} was recorded without dilution.

2.4 PCR Methodology for Identifying Unknown Microbial Strains from YPD Agar Plates

DNA extraction, PCR amplification, and primer selection were performed following a modified version of the protocol described by Kurtzman & Robnett (1997).

Sample Collection & DNA Extraction

1. A well-isolated colony of the unknown microbial strain was picked from YPD agar plates using a sterile loop.
2. 50 µL of freshly prepared 50 mM NaOH was pipetted into a sterile PCR tube or microcentrifuge tube.
3. The isolated colony was transferred into the NaOH solution by swirling the loop in the liquid to release cells.
4. The mixture was vortexed briefly (5–10 sec) to disperse cells.
5. The tube was then heated at 95°C for 10 min to lyse cells and release DNA.
6. After heating, the sample was cooled on ice for 2 min.
7. The lysate was centrifuged at 12,000 × g for 1–2 min, and the supernatant containing DNA was carefully pipetted into a new sterile tube.
8. The DNA supernatant (2–5 µL) was used as the template DNA for PCR.

Table 1. All the components and volumes to run one sample through PCR. These Components and volumes were replicated for each sample, each sample were run in triplicates, one triplicate with primers identifying bacteria and another triplicate with primers identifying yeasts.

Components	Volume
2x DreamTaq Green Master Mix	12.5 uL
Milli-Q water	8.5 uL
Forward primer	1 uL
Reverse primer	1 uL
DNA template	2 uL
Total volume	25 uL
The reaction mixture was thoroughly homogenized by gentle inversion and vortexing to ensure complete mixing of reagents.	

The primers for bacterial identification were primer 16Sf with the sequence of AGAGTTTGATCCTGGCTC and 16Sr with the sequence CGGGAACGTATTCACCG, the primers for yeast identification were NL1 with the sequence GCAGAGCAAGAAGCGGAGGAAAAG and NL4 with the sequence GGTCCGTGTTTCAAGACG (table 1).

Gel electrophoresis methodology to check validity of PCR products.

Gelperatation

A 1.5% agarose gel was prepared by dissolving 0.75 g of agarose in 50 mL of 1× TAE buffer. To facilitate DNA staining, 4.5 µL of GelRed Nucleic Acid Stain (10,000×) was added to the solution. The agarose solution was heated in a microwave until boiling was observed, ensuring complete dissolution of the agarose, and subsequently cooled to approximately 50 °C. The molten agarose was then poured into a casting tray fitted with a comb, and allowed to solidify at room temperature for approximately 20–30 minutes.

Sample loading

After gel solidification, the comb was carefully removed and the gel was placed in an electrophoresis chamber containing 1× TAE buffer. A molecular size standard (2.5 µL of GeneRuler Plus 1 kb DNA ladder) was loaded into one well, and 5 µL of each PCR-amplified sample was loaded into the remaining wells.

Electrophoresis and Visualization

The gel was run at 90–110 V until adequate separation of the DNA fragments was achieved, as judged by the migration of the loading dye. DNA bands were visualized under UV illumination using a gel documentation system to confirm PCR product size and quality.

Sequencing methodology

To estimate DNA concentration prior to sequencing, a Qubit™ Broad Range Fluorometer was used to measure a subset of randomly selected PCR products, providing an overview of the general concentration. PCR products that exhibited clearly visible bands under UV light and were presumed to have sufficient DNA concentration were selected, packaged, and sent to **Macrogen Europe BV** for purification and Sanger sequencing.

Strain 5 PCR products were aliquoted into tubes labeled **SD-01** and **SD-03**, while Strain 4 was pipetted into **SD-02** and **SD-04**. Sequencing was performed by Macrogen using standard Sanger sequencing protocols.

The resulting sequences were approximately **550–600 base pairs** for yeast samples and **1300-1500 base pairs** for bacterial samples. All sequences were analyzed using the **BLASTn** algorithm against the **NCBI GenBank** database to determine taxonomic identity based on sequence similarity (Altschul et al., 1990).

2.5 Bioinformatic Analysis of the *Streptomyces seoulensis* Genome

The bioinformatic pipeline was designed to functionally annotate the genome of *Streptomyces seoulensis* and identify enzymes potentially involved in yeast cell wall degradation. The analysis was performed in the following steps:

Genome Data Acquisition
The whole-genome sequence of *Streptomyces seoulensis* was obtained in FASTA format as an unannotated nucleotide file. This raw genomic data served as the input for downstream annotation and protein prediction.

Genome Annotation with Prokka
The unannotated genome was uploaded to the **Galaxy web platform** and analyzed using **Prokka**, an automated prokaryotic genome annotation tool. Prokka predicted coding sequences (CDSs), tRNAs, rRNAs, and other genomic elements, assigning gene names and functional annotations based on similarity to known sequences in curated reference databases such as UniProt and RefSeq. The annotation process produced multiple output files, including:

- .gff and .gbk (structural and feature annotation files),
- .faa (protein sequences in FASTA format),
- .tsv and .txt (tabulated gene and product descriptions).

Protein Extraction and Filtering
The .faa file was used to retrieve all predicted protein sequences encoded by the genome. Annotation tables (.txt/.tsv) were imported into Microsoft Excel, where proteins were filtered based on functional keywords in their product descriptions (e.g., “glucanase,” “chitinase,” “mannanase,” “protease,” “phospholipase”). Proteins were categorized based on their predicted roles in yeast cell wall degradation, and Enzyme Commission (EC) numbers were included to support functional classification when such annotations were available.

Functional Validation via BLAST
Selected protein sequences were submitted to the **NCBI BLASTP** tool to validate annotations. Sequences were compared against the non-redundant protein database to confirm predicted enzymatic functions based on sequence identity, e-value, and conserved domains.

CAZy Family Assignment Using dbCAN2
To further classify carbohydrate-active enzymes, candidate protein sequences were uploaded to the **dbCAN2 web server**. This tool integrates three algorithms—HMMER, DIAMOND, and Hotpep—to assign proteins to **CAZy families** such as glycoside hydrolases (GHs), glycosyltransferases (GTs), and carbohydrate esterases (CEs). Protein hits were examined for known GH families related to mannan and glucan degradation (e.g., GH76, GH92, GH125).

Organization and Visualization

Identified enzymes were categorized into five functional groups based on their predicted activity: glucanases, chitinases, mannanases, proteases, and GPI-cleaving enzymes. Results were summarized in tables for inclusion in the Results section, and full annotations and sequences were documented in the appendix and supplementary Excel files.

2.6 Biomass Quantification via Dry Cell Weight (DCW)

1. Prepare the Pre-Culture

1. **Inoculate a single colony** of the strain to be investigated into circa **20 mL of cell wall media** in a sterile mini flask during sterile conditions.
2. Incubate at 25°C, 150 RPM for 24 hours.
3. **Measure OD_{600}** of the pre-culture **using a spectrophotometer**.

2. Calculate Initial starting Concentration

- Use $\mathbf{C_1V_1 = C_2V_2}$ to determine how much pre-culture and cell wall media to transfer to achieve a desired starting OD_{600} in the mini flasks (see Example 1 in section 2.3 for reference).

3. Inoculation into Six Mini Flasks

1. Three flasks go **into the cultivation cabinet** (25°C, 150 RPM, 4 days, T=4).
2. Three flasks are **harvested immediately** (T = 0).

4. Biomass Harvesting (Initial & After 4 Days)

At T=0 (Immediate Harvesting)

1. **Pre-weigh empty centrifuge tubes**. Label them before weighing.
2. Pour the liquid culture from the mini flasks into 50 ml falcon tubes.
3. **Centrifuge cultures at 7000 x g for 10 min at 4°C** to pellet biomass. Remember to balance the centrifuge before centrifugation.
4. **Wash pellet 1×** with sterile water. Through another round of centrifugation.
5. **Carefully remove supernatant** and store the falcon tubes **in freezer to prepare for Freeze-drying (lyophilize) at -50°C**.

After 4 Days (Final Biomass Measurement)

1. Retrieve the remaining three flasks from the cultivation cabinet.
2. Perform the same steps as for T=0: weigh empty tubes, centrifuge at 7000 × g for 10 minutes at 4 °C, wash the pellet once with sterile water, discard the supernatant, and freeze the samples prior to freeze-drying.
3. Biomass was determined after lyophilization (freeze-drying) to ensure moisture-free dry weight measurements.

5. Data Analysis & Comparison

- Biomass yields (g/L) at T=0 and T=4 days were compared to assess microbial growth over the incubation period. Freeze-drying was used to eliminate moisture and ensure accurate dry biomass quantification.

Samples were freeze dried under vacuum in Scanvac coolsafe freeze drier at a temperature of -100°C for about 3 days.

Post-drying Measurement:
After freeze-drying, weigh the Falcon tubes and record the results. Subtract the weight of the empty tube from the falcon tubes plus biomass to calculate the dry cell weight (DCW). Compare the DCW from point zero to time point four to assess whether any cell growth occurred.

Results

This section presents the experimental results obtained from microbial growth monitoring using OD_{600} absorbance, biomass quantification, strain identification, and bioinformatic genome analysis of strain 5.
Growth kinetics of ten microbial isolates cultured on *Rhodotorula toruloides* cell wall media were monitored over a 7-day period using OD_{600} measurements (figure 2).
Biomass experiments were conducted in which the dry weight of microbial cultures was measured at day 0 and after 4 days of incubation to assess and compare the growth potential of each strain. The two strains that demonstrated the most robust growth from the microbial growth kinetics study (table 2 and figure 2) were selected for 16S rRNA gene sequencing to enable taxonomic identification. Subsequently, a comprehensive **in silico** genome analysis of *Streptomyces seoulensis* (strain 5) was conducted using bioinformatics tools to identify enzymes potentially involved in yeast cell wall degradation, with emphasis on glucanases, chitinases, mannanases, proteases, and GPI-cleaving enzymes. The results are organized into four sections: microbial growth and biomass data, molecular identification by 16S rRNA-gene sequencing and BLAST, and functional genome annotation.

3.1 Microbial Growth Monitoring Using OD_{600} Absorbance

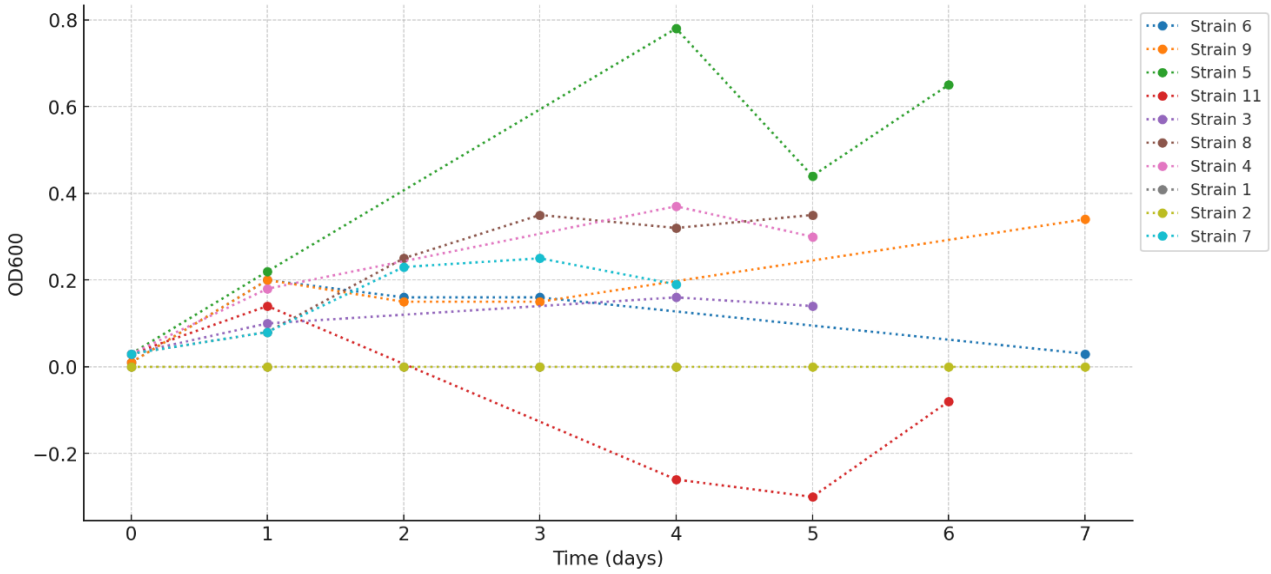

Figure 2. Growth curve graph showing the OD_{600} measurements over time for each strain. Strain 5 showed the highest OD_{600} values followed by strain 4 and 8.

The graph displays OD_{600} values measured at discrete time points (days 0, 1, 2, 3, 4, 5, and 7) for each strain, with dotted lines connecting only the actual measurements of each strain to the dots which is the measurement data. Strain 5 exhibited the highest growth, reaching an OD_{600} peak of approximately 0.8 on day 4, indicating strong biomass accumulation. In contrast, strains such as Strain 2 and Strain 3 showed minimal to no growth throughout the experimental period. Strain 11 demonstrated a temporary decline in growth between days 1 and 5 before partially recovering.

Table 2. Recorded OD_{600} values across measured time points for ten different microbial strains cultured over a 7-day period.

Time (days)	Strain 6	Strain 9	Strain 5	Strain 11	Strain 3	Strain 8	Strain 4	Strain 1	Strain 2	Strain 7
0	0.01	0.01	0.03	0.03	0.03	0.03	0.03	0	0	0.03
1	0.2	0.2	0.22	0.14	0.1	0.08	0.18	0	0	0.08
2	0.16	0.15					0.25	0	0	0.23
3	0.16	0.15					0.35	0	0	0.25
4			0.78	-0.26	0.16	0.32	0.37	0	0	0.19
5			0.44	-0.3	0.14	0.35	0.30			
6			0.65	-0.08						
7	0.03	0.34								

This table displays the corrected OD_{600} values for each strain measured at discrete time points (days 0 to 7), used to assess microbial growth dynamics in liquid culture. Initial OD_{600} values ranged between 0.01 and 0.03 across all strains. Strain 5 exhibited the most pronounced growth, reaching a peak OD_{600} of 0.78 on day 4. Strains such as Strain 4 and Strain 8 also showed steady increases in biomass. In contrast, Strains 1 and 2 displayed no measurable growth throughout the experiment, while Strain 11 showed a negative trend in OD, indicating potential cell death or measurement artifact. Missing values reflect days where no measurement was recorded.

3.2 Assessment of Biomass Production After 4-Day Incubation

To further quantify and assess the growth potential of the microbial strains, a biomass analysis was conducted by measuring the dry cell weight (DCW) of each culture at day 0 and after 4 days of incubation. The following figures and tables present the DCW results for strains that achieved the required minimum starting concentration ($OD_{600} \geq 0.05$) during the pre-culture phase.

Several strains—specifically Strains 1, 2, 3, 6, 9, and 11—did not meet this threshold and were therefore excluded from the biomass quantification experiment. This is consistent with the microbial growth monitoring study based on OD_{600} measurements, where Strains 1, 2, 3, 6, and 11 showed little to no growth throughout the 7-day period. Strain 9 initially displayed poor growth during the first 3 days, but a late-stage increase was observed in the OD_{600} data; however, strain 9 had to low OD_{600} values to meet the biomass experiment's entry criteria (table 2).

Strain 5 had biomass increasing from approximately 0.0012 g at day 0 to 0.0298 g on day 4. This growth confirms the strain's ability to utilize the *R. toruloides* cell wall fractions for biomass production, consistent with OD_{600} observations.

Table 3. Mean dry cell weight (DCW) of Strain 5 at time points 0 and 4. The mean DCW was measured over three biological replicates (n = 3), with error terms (±) indicating standard deviations.

Time points	Mean DCW (g)
0	0.001235 ± 0.00742
4	0.0298 ± 0.00028

The data in table 3 indicate an increase in biomass from 0.001235 g (± 0.00742 g) to 0.0298 g (± 0.00028 g) over 4 days, corresponding to approximately a 24-fold

increase. This demonstrates active growth of Strain 5 on the provided substrate during the incubation period.

Strain 5 had a DCW difference between time point 0 and 4 of 0,01745 g. Strain 5 grew 0,01745 grams during a 4-day period of incubation at 150 rpm at 25°C.

Table 4. Mean dry cell weight (DCW) of Strain 4 at time points 0 and 4. The mean DCW at time point one only has one biological measurement, the mean DCW at time point four has three biological measurements. With error terms (±) indicating standard deviations.

Time points	Mean DCW (g)
0	0.0026
4	0.0166 ± 0.00921

The table shows the biomass weight of Strain 4 over a 4-day incubation period, as measured by dry cell weight (DCW). At time point 0, the initial DCW was 0.0026 g, which increased to 0.0166 g at time point 4. This demonstrates that Strain 4 was able to utilize the growth medium to support active biomass accumulation. The increase in DCW is consistent with the strain's performance in the OD_{600}-based microbial growth monitoring study, where Strain 4 also showed steady growth over time (table 2).

Table 5. Mean dry cell weight (DCW) of Strain 8 measured at day 0 and after 4 days of incubation. The mean dry cell weight at time point 0 only had one biological measurement, the mean dry cell weight at time point 4 had three biological measurements. With error terms (±) indicating standard deviations.

Time points	Mean DCW (g)
0	0.0106
4	0.0128 ±0.0032

The results presented in Table 5 indicate a minor increase in the dry cell weight (DCW) of Strain 8 over the 4-day incubation period, from 0.0106 g at day 0 to 0.0128 ± 0.0032 g at day 4. It should be noted that the DCW at day 0 was based on a single biological measurement, whereas day 4 included three biological replicates. These findings are consistent with OD_{600}-based measurements, which also suggested only moderate growth of Strain 8. Taken together, the data imply that although Strain 8 may have weakly utilized the provided substrate, the observed biomass increase cannot be conclusively attributed to growth because the variability in measurements at day 4 overlaps with the single measurement at day 0, making it difficult to distinguish true growth from measurement uncertainty.

Table 6. Mean dry cell weight (DCW) of Strain 7 measured at day 0 and after 4 days of incubation. Mean dry cell weight at time point 0 had three biological measurements, mean dry cell weight at time point 4 had three biological measurements. With error terms (±) indicating standard deviations.

Time point	Mean DCW (g)
0	0,025 ±0.0125
4	0,01543 ±0.0136

The results presented in Table 6 indicate a reduction in the dry cell weight (DCW) of Strain 7 from 0.025 g at day 0 to 0.01543 g at day 4. This decrease in biomass over the incubation period may reflect limited metabolic activity, potential cell death, or partial degradation of the biomass. The relatively high standard deviations (±0.0125 g and ±0.0136 g, respectively) further suggest considerable variability in the measurements, which could stem from technical inconsistencies. Nevertheless, the trend aligns with the OD_{600} measurements, supporting the interpretation that Strain 7 showed minimal or inconsistent growth and may have been unable to utilize the substrate effectively under the given conditions.

3.3 Molecular Identification Based on 16S rRNA Gene Analysis

To confirm the presence of bacterial DNA in Strain 5, PCR amplification targeting the 16S rRNA gene was performed using both bacterial and yeast-specific primers. The resulting PCR products were visualized via agarose gel electrophoresis, as shown below.

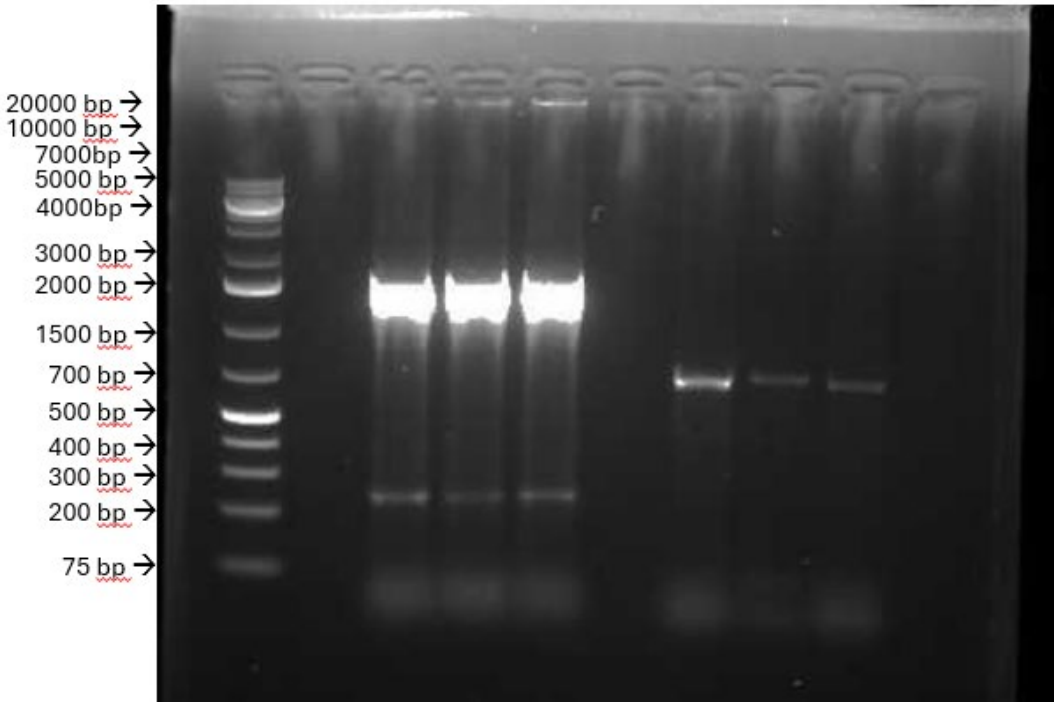

Figure 3. Agarose gel electrophoresis of PCR products from Strain 5 using bacterial and yeast identification primers.

PCR amplification using bacterial 16S rRNA primers (27F and 1492R) resulted in clearly visible DNA bands in wells 3, 4, and 5. These bands are approximately 1400–1500 base pairs (bp) in size, which aligns with the expected amplicon length for the 16S rRNA gene using this primer pair (Santosa et al., 2024) and (Frank et al., 2008). The presence of strong, size-consistent bands confirms successful amplification and the presence of bacterial DNA in Strain 5.

Only faint bands were observed in wells 7, 8, and 9, which were loaded with PCR products amplified using yeast-specific primers. These weak bands appeared between the 500 bp and 700 bp markers of the DNA ladder, consistent with the partial amplification of the D1/D2 region of the 26S rRNA gene in yeasts, which typically yields a fragment of approximately 600 bp as described by Kurtzman and Robnett (1997). This fragment size matches the DNA observed in wells 7, 8, and 9, indicating the presence of yeast DNA in these samples. The low intensity of these bands compared to the strong signals in wells 3, 4, and 5 suggests a relatively low abundance of yeast DNA in the sample, potentially indicating minor contamination of Strain 5 with yeasts.

A molecular weight marker (DNA ladder) was loaded in well 1, providing a reliable size reference for interpreting the results. The clarity and size of the observed bands confirm the specificity of the primers and the effectiveness of the electrophoresis procedure.

Taxonomic Identification via 16S rRNA Sequencing

The 16S rRNA gene sequences of the isolated bacterial strains were analyzed using BLASTn against the NCBI nucleotide database to determine their taxonomic identity. PCR products from Strain 4 and 5 were sequenced via Sanger sequencing by Macrogen Europe BV. The resulting nucleotide sequences were then submitted to BLASTn, and the top alignment hits were used to confirm species-level identification.

Identification of Strain 5 by BLAST Analysis

The 16S rRNA gene sequence of strain 5 was subjected to BLASTn analysis against the NCBI nucleotide database to determine its taxonomic affiliation. The BLAST searches consistently revealed that strain 5 is closely related to species within the genus *Streptomyces*, with highly significant alignment scores.

All top hits exhibited 100% query coverage, E-values of 0.0, and percentage identity ranging from 99.35% to 99.87%, indicating a strong phylogenetic relationship with the identified strains. Notably, the highest similarity was observed with *Streptomyces recifensis* strains (including strain LZ13-16 and strain TU22), *Streptomyces sp. G6-13*, *Streptomyces sp. NBC_01171*, and *Streptomyces seoulensis* (see figure 6 and table 7). Several complete genomes, such as those of *Streptomyces sp. NBC_01171*, *Streptomyces sp. NBC_01601*, *Streptomyces seoulensis* and *Streptomyces sp. NBC_00568*, also appeared among the top matches, providing additional confirmation of strain 5's classification within the genus *Streptomyces* (see Figure 6 in the appendix, where a match with the *Streptomyces seoulensis* complete genome was observed).

Although strain 5 did not show 100% sequence identity to any single strain, the observed high similarity (>99%) suggests that it is a close relative of these *Streptomyces* species, possibly representing a distinct strain or a natural variant. The E-value represents the expected number of matches with a similar score that could occur by chance, with lower values indicating higher confidence in the sequence match. An E-value of 0.0 indicates that the match is highly significant and extremely unlikely to have occurred by random chance.

Table 7. A summary of the most significant BLAST hits is presented in.

Description	Scientific Name	Query Coverage (%)	% Identity	E-value	Accession Number
Streptomyces recifensis strain LZ13-16 16S	Streptomyces recifensis	100	99.36	0.0	MT586247.1

rRNA gene, partial sequence					
Streptomyces sp. G6-13 16S rRNA gene, partial sequence	*Streptomyces sp.* G6-13	100	99.36	0.0	JN866756.1
Streptomyces recifensis strain TU22 16S ribosomal RNA gene, partial sequence	*Streptomyces recifensis*	100	99.36	0.0	MH482880.1
Streptomyces sp. strain BS-3 16S ribosomal RNA gene, partial sequence	*Streptomyces sp.*	100	99.36	0.0	OQ438813.1
Streptomyces sp. strain XKC-SO-4 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	100	99.36	0.0	OQ456539.1
Streptomyces sp. strain XKC-SO-16 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	100	99.36	0.0	OQ456534.1

The BLAST analysis of strain 5's 16S rRNA gene sequence revealed high similarity to multiple *Streptomyces* species, with 100% query coverage and percentage identities of 99.36% across all top hits. The closest matches included *Streptomyces recifensis* strains (LZ13-16 and TU22) and other *Streptomyces* species such as *Streptomyces sp. G6-13*, *Streptomyces sp.* strain BS-3, *Streptomyces sp.* strain XKC-SO-4, and *Streptomyces sp.* strain XKC-SO-16. These results confirm that strain 5 is closely related to members of the genus *Streptomyces (table 2)*.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Streptomyces recifensis strain LZ13-16 16S ribosomal RNA gene, partial sequence	Streptomyces recifensis	1683	1683	100%	0.0	99.36%	1430	MT856247.1
Streptomyces sp. G6-13 16S ribosomal RNA gene, partial sequence	Streptomyces sp. G6-13	1683	1683	100%	0.0	99.36%	1405	JN866756.1
Streptomyces recifensis strain TU22 16S ribosomal RNA gene, partial sequence	Streptomyces recifensis	1683	1683	100%	0.0	99.36%	1425	MH482880.1
Streptomyces sp. strain BS-3 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1683	1683	100%	0.0	99.36%	1456	OQ438813.1
Streptomyces sp. strain XKC-SO-4 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1683	1683	100%	0.0	99.36%	1456	OQ449531.1
Streptomyces sp. strain XKC-SO-16 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1683	1683	100%	0.0	99.36%	1465	OQ449539.1
Streptomyces sp. strain LK-R-7 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1683	1683	100%	0.0	99.36%	1462	OQ456524.1
Streptomyces sp. NBC_01171 chromosome, complete genome	Streptomyces sp. NBC_01171	1681	10089	100%	0.0	99.35%	6584968	CP108609.1
Streptomyces sp. NBC_01601 chromosome, complete genome	Streptomyces sp. NBC_01601	1681	10089	100%	0.0	99.35%	6514704	CP109314.1
Streptomyces sp. strain A1314 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1681	1681	100%	0.0	99.35%	1010	KU865524.1
Streptomyces sp. strain A1303 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1681	1681	100%	0.0	99.35%	1011	KU865516.1
Streptomyces sp. NBC_00566 chromosome, complete genome	Streptomyces sp. NBC_00566	1681	10089	100%	0.0	99.35%	6411420	CP107784.1
Streptomyces sp. 6IS-1 16S ribosomal RNA gene, partial sequence	Streptomyces sp. 6IS-1	1681	1681	100%	0.0	99.35%	1402	JN866761.1
Streptomyces seoulensis HEK131 DNA, complete genome	Streptomyces seoulensis	1681	10083	100%	0.0	99.35%	6542274	AP025667.1
Streptomyces seoulensis strain HBUM173318 16S ribosomal RNA gene, partial sequence	Streptomyces seoulensis	1681	1681	100%	0.0	99.35%	1449	EU841610.1

Figure 4. BLASTn results of the 16S rRNA gene sequence from strain 5 aligned against the NCBI nucleotide database. The top alignment hits display 100% query coverage and ≥99.35% sequence identity to various *Streptomyces* species, including *Streptomyces seoulensis* (Accession: AP025667.1). These high-confidence matches support the classification of strain 5 within the *Streptomyces* genus and specifically as a close relative of *S. seoulensis*. This sequence similarity provided the rationale for conducting a genome annotation using *S. seoulensis* as the reference framework in downstream functional analyses.

This sequence match provided the biological rationale for conducting a **Prokka genome annotation** specifically using the *S. seoulensis* complete genome as reference framework. By aligning the genome of strain 5 to a validated *S. seoulensis* genome, we ensured accurate functional prediction and enzyme identification related to yeast cell wall degradation. The high sequence identity validated the genome's origin and justified using CAZy and EC-based annotation approaches to explore its enzymatic repertoire.

Yeast Cell Wall-Degrading Enzymes in *Streptomyces seoulensis*

Table 8. Annotated proteins from the Streptomyces genome that are potentially involved in yeast cell wall degradation. These enzymes target key structural components such as β-glucans, chitin, mannans, GPI-anchored proteins, and polypeptide backbones. Each enzyme entry includes its locus tag, name, EC number (if available), and literature reference supporting its function.

Locus Tag	Gene	Product	EC Number	Enzyme Group	Reference
DOODJJCJ_00060	-	Glucan endo-1,3-beta-glucosidase	3.2.1.39	Glucanases	Chen et al., 2003 (https://doi.org/10.1128/jb.17

					9.19.6028-6034.1997)
DOODJJCJ_00061	bglA_1	Beta-glucanase	3.2.1.73	Glucanases	Planas, 2000 (https://doi.org/10.1128/jb.179.19.6028-6034.1997)
DOODJJCJ_00088	-	Glucan endo-1,3-beta-glucosidase	3.2.1.39	Glucanases	Chen et al., 2003 (https://doi.org/10.1128/jb.179.19.6028-6034.1997)
DOODJJCJ_00106	glgB_1	1,4-alpha-glucan branching enzyme GlgB	2.4.1.18	Glucanases	Uniprot, 2024 (https://www.uniprot.org/uniprotkb/P07762/entry)
DOODJJCJ_01577	malL_1	Oligo-1,6-glucosidase	3.2.1.10	Glucanases	N/A
DOODJJCJ_01756	ygjK	Glucosidase YgjK	3.2.1.-	Glucanases	N/A
DOODJJCJ_01793	bglA_2	Beta-glucosidase A	3.2.1.21	Glucanases	N/A
DOODJJCJ_01911	malQ	4-alpha-glucanotransferase	2.4.1.25	Glucanases	N/A
DOODJJCJ_01920	licH_1	putative 6-phospho-beta-glucosidase	3.2.1.86	Glucanases	N/A
DOODJJCJ_02047	-	Bifunctional beta-D-glucosidase/beta-D-fucosidase	3.2.1.21	Glucanases	N/A
DOODJJCJ_02079	cenA	Endoglucanase A	3.2.1.4	Glucanases	N/A
DOODJJCJ_03002	malL_2	Oligo-1,6-glucosidase	3.2.1.10	Glucanases	N/A
DOODJJCJ_04020	glgB_2	1,4-alpha-glucan branching enzyme GlgB	2.4.1.18	Glucanases	Uniprot, 2024 (https://www.uniprot.org/uniprotkb/Q2P949/entry)
DOODJJCJ_04804	bglB	Beta-glucosidase B	3.2.1.21	Glucanases	N/A
DOODJJCJ_04831	licH_2	putative 6-phospho-beta-glucosidase	3.2.1.86	Glucanases	N/A
DOODJJCJ_04843	malL_3	Oligo-1,6-glucosidase	3.2.1.10	Glucanases	N/A
DOODJJCJ_05344	celS	Endoglucanase S	3.2.1.4	Glucanases	N/A

DOODJJCJ_03665	-	Exochitinase 1	3.2.1.14	Chitinases	N/A
DOODJJCJ_03960	chiC	Chitinase C	3.2.1.14	Chitinases	N/A
DOODJJCJ_04221	-	Exochitinase 1	3.2.1.14	Chitinases	N/A
DOODJJCJ_05568	chiA2	Chitinase	3.2.1.14	Chitinases	N/A
DOODJJCJ_00007	dpp5	Dipeptidyl-peptidase 5	3.4.14.-	Proteases	N/A
DOODJJCJ_00084	dap_1	D-aminopeptidase	3.4.11.19	Proteases	N/A
DOODJJCJ_00089	-	Transglutaminase-activating metalloprotease	3.4.-.-	Proteases	N/A
DOODJJCJ_00121	pepN_1	Aminopeptidase N	3.4.11.2	Proteases	N/A
DOODJJCJ_00131	-	Transglutaminase-activating metalloprotease	3.4.-.-	Proteases	N/A
DOODJJCJ_00132	-	D-alanyl-D-alanine carboxypeptidase	3.4.16.4	Proteases	N/A
DOODJJCJ_00234	-	Transglutaminase-activating metalloprotease	3.4.-.-	Proteases	N/A
DOODJJCJ_00242	ftsI	Peptidoglycan D,D-transpeptidase FtsI	3.4.16.4	Proteases	N/A
DOODJJCJ_00248	dap_2	D-aminopeptidase	3.4.11.19	Proteases	N/A
DOODJJCJ_00254	mrdA_1	Peptidoglycan D,D-transpeptidase MrdA	3.4.16.4	Proteases	N/A
DOODJJCJ_00277	htpX_1	Protease HtpX	3.4.24.-	Proteases	N/A
DOODJJCJ_00308	pip	Proline iminopeptidase	3.4.11.5	Proteases	N/A
DOODJJCJ_00487	vanX	D-alanyl-D-alanine dipeptidase	3.4.13.22	Proteases	N/A
DOODJJCJ_00511	-	Serine protease inhibitor	nan	Proteases	N/A
DOODJJCJ_00553	ftsH_1	ATP-dependent zinc metalloprotease FtsH	3.4.24.-	Proteases	N/A
DOODJJCJ_00588	yraA	Putative cysteine protease YraA	3.2.-.-	Proteases	N/A
DOODJJCJ_00608	ddpX	D-alanyl-D-alanine dipeptidase	3.4.13.22	Proteases	N/A
DOODJJCJ_00609	pepN_2	Aminopeptidase N	3.4.11.2	Proteases	N/A

DOODJJCJ_00641	tap_1	Tripeptidyl aminopeptidase	3.4.14.-	Proteases	N/A
DOODJJCJ_00649	clpP2_1	ATP-dependent Clp protease proteolytic subunit 2	3.4.21.92	Proteases	N/A
DOODJJCJ_00829	-	Serine protease inhibitor	nan	Proteases	N/A
DOODJJCJ_00857	-	putative peptidase	3.4.-.-	Proteases	N/A
DOODJJCJ_00999	-	Serine protease inhibitor	nan	Proteases	N/A
DOODJJCJ_01188	tldD	Metalloprotease TldD	3.4.-.-	Proteases	N/A
DOODJJCJ_01369	pepN_3	Aminopeptidase N	3.4.11.2	Proteases	N/A
DOODJJCJ_01373	glpG_1	Rhomboid protease GlpG	3.4.21.105	Proteases	N/A
DOODJJCJ_01408	lon_1	Lon protease	3.4.21.53	Proteases	N/A
DOODJJCJ_01426	lspA	Lipoprotein signal peptidase	3.4.23.36	Proteases	N/A
DOODJJCJ_01505	ldtB_1	L,D-transpeptidase 2	2.3.2.-	Proteases	N/A
DOODJJCJ_01536	pepA	putative cytosol aminopeptidase	3.4.11.1	Proteases	N/A
DOODJJCJ_01543	-	Serine protease	3.4.21.-	Proteases	N/A
DOODJJCJ_01559	htpX_2	Protease HtpX	3.4.24.-	Proteases	N/A
DOODJJCJ_01606	rip2	Putative zinc metalloprotease Rip2	nan	Proteases	N/A
DOODJJCJ_01611	map	Methionine aminopeptidase 2	3.4.11.18	Proteases	N/A
DOODJJCJ_01619	yfeW_1	Putative D-alanyl-D-alanine carboxypeptidase	3.4.16.4	Proteases	N/A
DOODJJCJ_01687	yfeW_2	Putative D-alanyl-D-alanine carboxypeptidase	3.4.16.4	Proteases	N/A
DOODJJCJ_01723	-	Zinc D-Ala-D-Ala carboxypeptidase	3.4.17.14	Proteases	N/A
DOODJJCJ_01747	-	Transglutaminase-activating metalloprotease	3.4.-.-	Proteases	N/A
DOODJJCJ_01791	prtS	Protease PrtS	3.4.24.-	Proteases	N/A

DOODJJCJ_01812	tri1_2	Tricorn protease	3.4.21.-	Proteases	N/A
DOODJJCJ_01848	htpX_3	Protease HtpX	3.4.24.-	Proteases	N/A
DOODJJCJ_01873	mrdA_2	Peptidoglycan D,D-transpeptidase MrdA	3.4.16.4	Proteases	N/A
DOODJJCJ_01882	clpX	ATP-dependent Clp protease ATP-binding subunit ClpX	nan	Proteases	N/A
DOODJJCJ_01883	clpP2_2	ATP-dependent Clp protease proteolytic subunit 2	3.4.21.92	Proteases	N/A
DOODJJCJ_01884	clpP1	ATP-dependent Clp protease proteolytic subunit 1	3.4.21.92	Proteases	N/A
DOODJJCJ_01900	pepN_4	Aminopeptidase N	3.4.11.2	Proteases	N/A
DOODJJCJ_01905	pepN_5	Aminopeptidase N	3.4.11.2	Proteases	N/A
DOODJJCJ_02006	ptp	Prolyl tri/tetrapeptidyl aminopeptidase	3.4.11.-	Proteases	N/A
DOODJJCJ_02118	mec	CysO-cysteine peptidase	3.13.1.6	Proteases	N/A
DOODJJCJ_02121	clpS	ATP-dependent Clp protease adapter protein ClpS	nan	Proteases	N/A
DOODJJCJ_02125	prtV	Pre-pro-metalloprotease PrtV	3.4.24.-	Proteases	N/A
DOODJJCJ_02134	lon_2	Lon protease	3.4.21.53	Proteases	N/A
DOODJJCJ_02347	mrdA_3	Peptidoglycan D,D-transpeptidase MrdA	3.4.16.4	Proteases	N/A
DOODJJCJ_02348	mrdA_4	Peptidoglycan D,D-transpeptidase MrdA	3.4.16.4	Proteases	N/A
DOODJJCJ_02353	dap_3	D-aminopeptidase	3.4.11.19	Proteases	N/A
DOODJJCJ_02361	tri1_3	Tricorn protease	3.4.21.-	Proteases	N/A
DOODJJCJ_02811	pepPI	Xaa-Pro aminopeptidase 1	3.4.11.9	Proteases	N/A
DOODJJCJ_02855	dap_4	D-aminopeptidase	3.4.11.19	Proteases	N/A
DOODJJCJ_02905	glpG_2	Rhomboid protease GlpG	3.4.21.105	Proteases	N/A
DOODJJCJ_02969	-	D-alanyl-D-alanine carboxypeptidase	3.4.16.4	Proteases	N/A

DOODJJCJ_02980	apeB	putative M18 family aminopeptidase 2	3.4.11.-	Proteases	N/A
DOODJJCJ_03129	-	Serine protease	3.4.21.-	Proteases	N/A
DOODJJCJ_03180	ftsH_2	ATP-dependent zinc metalloprotease FtsH	3.4.24.-	Proteases	N/A
DOODJJCJ_03207	clpC1_1	ATP-dependent Clp protease ATP-binding subunit ClpC1	nan	Proteases	N/A
DOODJJCJ_03357	htpX_4	Protease HtpX	3.4.24.-	Proteases	N/A
DOODJJCJ_03415	map-1_1	Methionine aminopeptidase 1	3.4.11.18	Proteases	N/A
DOODJJCJ_03624	ldtB_2	L,D-transpeptidase 2	2.3.2.-	Proteases	N/A
DOODJJCJ_03750	dap4	Dipeptidyl aminopeptidase 4	3.4.14.5	Proteases	N/A
DOODJJCJ_03898	clpC1_2	ATP-dependent Clp protease ATP-binding subunit ClpC1	nan	Proteases	N/A
DOODJJCJ_03914	lon_3	Lon protease	3.4.21.53	Proteases	N/A
DOODJJCJ_04027	-	Transglutaminase-activating metalloprotease	3.4.-.-	Proteases	N/A
DOODJJCJ_04028	-	Transglutaminase-activating metalloprotease	3.4.-.-	Proteases	N/A
DOODJJCJ_04034	ldtB_3	L,D-transpeptidase 2	2.3.2.-	Proteases	N/A
DOODJJCJ_04035	lppS	Putative L,D-transpeptidase LppS	2.3.2.-	Proteases	N/A
DOODJJCJ_04108	-	Serine protease inhibitor	nan	Proteases	N/A
DOODJJCJ_04112	-	Serine protease inhibitor	nan	Proteases	N/A
DOODJJCJ_04231	rip1	Zinc metalloprotease Rip1	3.4.24.-	Proteases	N/A
DOODJJCJ_04267	-	putative zinc protease	3.4.24.-	Proteases	N/A
DOODJJCJ_04390	-	putative zinc protease	3.4.24.-	Proteases	N/A
DOODJJCJ_04434	yfeW_3	Putative D-alanyl-D-alanine carboxypeptidase	3.4.16.4	Proteases	N/A
DOODJJCJ_04552	tap_4	Tripeptidyl aminopeptidase	3.4.14.-	Proteases	N/A

DOODJJCJ_04582	pepE_1	putative dipeptidase PepE	3.4.13.-	Proteases	N/A
DOODJJCJ_04586	sti	subtilase-type protease inhibitor	nan	Proteases	N/A
DOODJJCJ_04715	clpC_1	ATP-dependent Clp protease ATP-binding subunit ClpC	nan	Proteases	N/A
DOODJJCJ_04716	map-1_2	Methionine aminopeptidase 1	3.4.11.18	Proteases	N/A
DOODJJCJ_04727	alpha-LP	Alpha-lytic protease	3.4.21.12	Proteases	N/A
DOODJJCJ_04758	tap_5	Tripeptidyl aminopeptidase	3.4.14.-	Proteases	N/A
DOODJJCJ_04764	dppA	D-aminopeptidase	3.4.11.-	Proteases	N/A
DOODJJCJ_04767	ykfA	putative murein peptide carboxypeptidase	3.4.16.-	Proteases	N/A
DOODJJCJ_04884	pepN_6	Aminopeptidase N	3.4.11.2	Proteases	N/A
DOODJJCJ_04885	pepX	Xaa-Pro dipeptidyl-peptidase	3.4.14.11	Proteases	N/A
DOODJJCJ_05023	pepE_2	Peptidase E	3.4.13.21	Proteases	N/A
DOODJJCJ_05041	htpX_5	Protease HtpX	3.4.24.-	Proteases	N/A
DOODJJCJ_05221	bepA	Beta-barrel assembly-enhancing protease	3.4.-.-	Proteases	N/A
DOODJJCJ_05273	-	Serine protease inhibitor	nan	Proteases	N/A
DOODJJCJ_05562	-	Serine protease inhibitor	nan	Proteases	N/A
DOODJJCJ_05612	clpC_2	ATP-dependent Clp protease ATP-binding subunit ClpC	nan	Proteases	N/A
DOODJJCJ_05630	clpC_3	ATP-dependent Clp protease ATP-binding subunit ClpC	nan	Proteases	N/A
DOODJJCJ_05695	snpA_1	Extracellular small neutral protease	3.4.24.77	Proteases	N/A
DOODJJCJ_05696	snpA_2	Extracellular small neutral protease	3.4.24.77	Proteases	N/A
DOODJJCJ_00039	plcN_1	Non-hemolytic phospholipase C	3.1.4.3	GPI-cleaving enzymes	N/A
DOODJJCJ_01093	-	Lipase 1	3.1.1.3	GPI-cleaving enzymes	N/A

DOODJJCJ_01419	pld	Phospholipase D	3.1.4.4	GPI-cleaving enzymes	N/A
DOODJJCJ_01479	-	Thermostable monoacylglycerol lipase	3.1.1.23	GPI-cleaving enzymes	N/A
DOODJJCJ_03801	lip1	Lipase 1	3.1.1.3	GPI-cleaving enzymes	N/A
DOODJJCJ_04507	plcN_2	Non-hemolytic phospholipase C	3.1.4.3	GPI-cleaving enzymes	N/A
DOODJJCJ_05002	-	Monoacylglycerol lipase	3.1.1.23	GPI-cleaving enzymes	N/A
DOODJJCJ_05669	-	Lipase 2	3.1.1.3	GPI-cleaving enzymes	N/A

Table 8**.** The annotated enzymatic profile of *Streptomyces seoulensis* demonstrates a broad capacity for yeast cell wall degradation, primarily driven by a wide array of glucanases such as glucan endo-1,3-β-glucosidases and β-glucosidases, which target β-glucan polymers abundant in fungal walls. In addition, several chitinases and a large variety of proteases were identified, enabling potential hydrolysis of chitin and proteinaceous components of the wall. Multiple GPI-cleaving enzymes were also annotated, suggesting an ability to release GPI-anchored surface proteins. Notably, no mannan-degrading enzymes such as α-mannosidases were detected in the genome annotation, indicating a possible limitation in targeting the mannan layer. These findings highlight the enzymatic versatility of *S. seoulensis* and its potential utility in biotechnological processes involving fungal biomass deconstruction.

Identification of Strain 4 by BLAST Analysis

The amplified 16S rRNA gene sequence obtained from strain 4 was subjected to two independent BLASTn searches against the NCBI database to determine its phylogenetic affiliation. Both searches yielded consistent results, revealing that strain 4 shares a high degree of similarity with multiple *Streptomyces* species.

All top hits displayed:

- **100% query coverage**,
- **E-values of 0.0**, and
- **Percentage identity between 99.00% and 100.00%**,

suggesting that strain 4 belongs to the genus *Streptomyces*. The repeated identification of the same or highly related strains across both searches reinforces this conclusion.

Table 9. BLASTN results of 16S rRNA gene sequence from Strain 4 against the NCBI nucleotide database.

Description	Scientific Name	Query Coverage (%)	% Identity	E-value	Accession Number
Chromosome, complete genome	Streptomyces sp. NBC 01171	100	100.00	0.0	CP108809.1
16S ribosomal RNA gene, partial sequence	Streptomyces sp. LaPaAH181	100	100.00	0.0	KJ889048.1
16S ribosomal RNA gene, partial sequence	Streptomyces sp.	100	100.00	0.0	KX577388.1
16S ribosomal RNA gene, partial sequence	Streptomyces seoulensis	100	100.00	0.0	MN328857.1
Chromosome, complete genome	Streptomyces sp. NBC 00568	100	100.00	0.0	CP107784.1
16S ribosomal RNA gene, partial sequence	Streptomyces recifensis	100	99.00	0.0	MH482880.1

This table shows the top alignment hits from a BLASTN search using the 16S rRNA gene sequence amplified from Strain 4. All listed matches exhibited 100% query coverage and ≥99% sequence identity, with E-values of 0.0, indicating highly significant alignments. Among the top hits is *Streptomyces seoulensis* (Accession: MN328857.1), suggesting that Strain 4 is most likely a member of the *Streptomyces seoulensis* species. Additional top matches include other *Streptomyces* species and complete genome entries, further supporting its classification within the *Streptomyces* genus.

Discussion

This study assessed the yeast cell wall-degrading capabilities of ten microbial isolates using growth kinetics, biomass production, 16S rRNA sequencing, and genome-based enzyme annotation. Strain 5 (*Streptomyces* sp., closely related to *S. recifensis* and *S. seoulensis*) and Strain 4 (*Streptomyces seoulensis*, supported by 100% identity to reference sequences) emerged as the most promising candidates for further analysis, based on their high OD_{600} values and biomass production.

OD_{600} as a Proxy for Microbial Biomass Quantification

Optical density at 600 nm (OD_{600}) is widely employed as a rapid, non-destructive method to estimate microbial biomass concentration in liquid cultures. This technique measures turbidity caused by suspended cells, providing an indirect but reliable indication of cell density and cell concentration. Numerous studies have validated OD_{600} as a proxy for microbial biomass across different species. For example, Kim et al. (2012) demonstrated a strong linear correlation between OD_{600} values and both dry biomass and colony-forming units (CFU) in *Pseudomonas aeruginosa*, offering specific conversion equations (e.g., 1 $OD_{600} \approx$ 2.085 mg/mL dry weight). Similarly, Karamba and Ahmad (2019) confirmed the mathematical relationship between OD_{600} and microbial dry weight in *Serratia marcescens*, reinforcing its consistency across varying cell densities. Additionally, Ma (2011) highlighted OD_{600}'s utility in tracking biomass changes during yeast fermentations, where it effectively monitored growth phases and lysis events. These findings support the use of OD_{600} as a reliable and accessible tool for quantifying microbial concentration and evaluating the impact of enzymatic or chemical treatments in microbial cell studies.

Synergistic Enzyme Combinations for Enhanced Yeast Cell Wall Degradation

Enzyme mixtures derived from different microbial isolates can significantly enhance the degradation of yeast cell walls through synergistic action. While individual enzymes such as β-glucanases, chitinases, and proteases target specific cell wall components, their combined use can lead to more efficient and comprehensive lysis. Synergy arises when one enzyme's activity exposes substrates or creates access points for others, amplifying the overall degradation effect. This principle is supported by studies demonstrating that enzyme cocktails outperform single-enzyme treatments in breaking down complex polysaccharide structures. For instance, Zhang et al. (2024) showed that combining hydrolytic enzymes from *Aspergillus* species resulted in more effective lysis of fungal biomass than individual enzymes alone. Similarly, Machinandiarena et al. (2005) observed that fungal endoglucanases and proteases acted synergistically to degrade plant and microbial cell walls. These findings suggest that employing multi-enzyme systems could be a valuable strategy in biotechnological applications where efficient cell wall disruption is essential—such as lipid extraction, metabolite release, or bioproduct recovery from yeast biomass.

Microbial Growth and Biomass Accumulation

Among the tested unknown microbial isolates, the variability observed in microbial growth dynamics is often attributed to differences in substrate utilization, metabolic activity, and inherent growth rates among strains, as supported by direct single-cell tracking studies (McClelland et al., 2020). Strain 5 demonstrated the highest growth, reaching a peak OD_{600} of 0.78 on day 4, a trend that was mirrored in its corresponding increase in dry cell weight during the biomass quantification test. This suggests a strong capacity for substrate

utilization. Similar findings have been reported, where *Streptomyces* and *Acremonium* isolates effectively degraded yeast cell walls and facilitated protoplast formation in fungal species (Kaul et al., 1993). Moreover, *Streptomyces* species are well-documented to exhibit lytic activity against yeast cell walls, largely due to their secretion of glucanases, chitinases, and proteases (Tanaka and Phaff, 1965). These enzymatic characteristics are consistent with the high growth performance observed in Strain 5.

Strain 4 also showed notable biomass accumulation, supporting earlier observations that *Streptomyces* isolates derived from decaying organic environments often possess strong yeast-lytic activity (Chernyakovskaya et al., 2004). In contrast, strains such as Strain 7, which displayed poor or negative growth, may lack the enzymatic systems or regulatory mechanisms required for effective yeast cell wall degradation. This variability is consistent with earlier studies highlighting the dependence of lytic activity on microbial origin and enzyme composition (Knorr et al., 1979).

Identification and Bioinformatic Confirmation

Multiple BLASTn analyses were performed on the 16S rRNA sequence of Strain 5, which consistently returned high similarity scores (>99%) to both *Streptomyces seoulensis* and *S. recifensis*. Although the identity was not 100%, these results, together with genome-based enzyme annotations, support its tentative classification as *Streptomyces* sp. closely related to these species. In contrast, Strain 4 showed 100% identity to *S. seoulensis* in BLASTn results, justifying its assignment to this species. However, it is important to note that 16S rRNA sequencing alone may lack the resolution for definitive species-level classification among closely related actinobacteria, and future studies should consider whole-genome or multilocus sequence analyses to improve taxonomic accuracy.

Functional Classification of Yeast Cell Wall-Degrading Enzymes

Analysis of the *Streptomyces seoulensis* genome revealed a diverse suite of enzymes predicted to degrade the major structural components of the yeast cell wall. These enzymes were classified into five functional groups based on their substrate specificity: **glucanases**, **chitinases**, **mannanases**, **proteases**, and **GPI-cleaving enzymes** (table 10).

Table 10. Summary of Enzyme Classes Involved in Yeast Cell Wall Degradation.

Enzyme Class	Description	Source (DOI)
Glucanases	Enzymes that hydrolyze β-glucans, key polysaccharides in yeast cell walls, especially β-1,3 and β-1,6 linkages.	https://doi.org/10.1016/j.enzmictec.2021.109948
Chitinases	Break down chitin, a structural polymer of N-acetylglucosamine, prevalent in fungal inner walls.	https://doi.org/10.1094/Phyto-84-398
Mannanases	Degrade mannans and mannoproteins in the outer yeast wall by cleaving α-1,6- or α-1,2-mannosidic bonds.	https://doi.org/10.1016/j.carres.2007.07.020

Proteases	Hydrolyze peptide bonds in structural proteins like GPI-anchored or Pir proteins embedded in the yeast cell wall.	https://doi.org/10.1016/j.fgb.2011.05.005
GPI-cleaving enzymes	Target glycosylphosphatidylinositol (GPI) anchors, enabling release of surface-anchored proteins.	https://pmc.ncbi.nlm.nih.gov/articles/PMC4689333/

The most prevalent and functionally significant enzymes identified in *Streptomyces seoulensis* were glucanases, especially glucan endo-1,3-β-glucosidases (EC 3.2.1.39), which cleave internal β-1,3-glucan bonds—key structural elements of fungal and yeast cell walls. These enzymes play a central role in initiating cell wall lysis by breaking down the glucan matrix (Ma et al., 2021). Supporting their action, β-glucosidases (EC 3.2.1.73) were also present and are known to hydrolyze terminal residues on glucan chains, thereby enhancing glucan degradation when working synergistically with endo-acting enzymes (Igarashi et al., 2003).

Chitin-degrading enzymes were also well represented. Several chitinases (EC 3.2.1.14), which target β-1,4-linked N-acetylglucosamine in the chitin layer, were annotated. These enzymes are commonly involved in fungal antagonism and are useful in biocontrol strategies making them an attractive option for breakdown of the yeast cell wall, especially because they are eco-friendly (Lorito et al., 1994). Additional support for chitin breakdown came from the detection of exo-β-D-glucosaminidases (EC 3.2.1.165), which process chitin-derived oligosaccharides (Cutfield et al., 1999).
Although mannan-degrading enzymes were initially expected, the annotation revealed no α-mannosidases (EC 3.2.1.24) or other mannanases, suggesting a limited or absent capacity for cleaving mannoproteins in the outer yeast cell wall. In contrast, a broad range of proteases—including serine, zinc metalloproteases, and aminopeptidases—were identified. These enzymes likely contribute to the degradation of protein components within the cell wall, such as Pir proteins and GPI-anchored surface proteins, and may facilitate structural remodeling during lysis (Yike, 2011).

Furthermore, several lipase-like and phospholipase enzymes were found, which may target glycosylphosphatidylinositol (GPI) anchors. These enzymes are known to release GPI-bound proteins from the fungal surface and modulate yeast cell wall structure (Doering, 2000).

In summary, *Streptomyces seoulensis* encodes a broad suite of enzymes capable of targeting key structural components of the yeast cell wall, including β-glucans, chitin, proteins, and GPI-anchors (figure 1). Notably, while no mannanases were identified, the presence of multiple glucanases and chitinases, as well as proteases and GPI-cleaving enzymes, underscores the organism's potential for yeast cell wall degradation (Ma et al., 2021; Cutfield et al., 1999; Tanaka and Phaff, 1965). However, the exact yeast cell wall composition of *Rhodotorula toruloides* is still unknown. But the experiments done in this study confirm that *Streptomyces*

seoulensis and *Streptomyces* spp. have the capabilities to grow and multiply using *Rhodotorula toruloides* cell wall fractions as the sole carbon source in a nitrogen-limited minimal medium. The rationale for using a minimal medium with *Rhodotorula toruloides* cell wall material as the sole carbon source was to impose selective pressure that highlights the enzymatic capabilities of microbial isolates. In this medium, growth is only possible if the microorganism can degrade and metabolize complex cell wall components such as β-glucans, chitin, mannans, and glycoproteins. This approach enables the identification of microbial strains capable of degrading yeast cell wall components, not only through secreted extracellular yeast cell wall degrading enzymes but also potentially via cell wall-associated or intracellular yeast cell wall degrading enzymes (Peciarová & Biely, 1982), (Morgavi et al., 1994) and (Tanaka & Phaff, 1965).

Furthermore, the medium closely simulates industrially relevant conditions where enzymatic pretreatment is required to disrupt the yeast cell wall and release intracellular contents such as lipids. This is particularly important for the downstream processing of oleaginous yeasts like *R. toruloides*, which are increasingly explored for biofuel and oleochemical production (Zhao et al., 2021). Additionally, the use of a defined, minimal nutrient background ensures that any increase in biomass can be directly linked to the utilization of yeast cell wall components. The absence of alternative carbon sources minimizes experimental noise, allowing clearer interpretation of enzymatic activity and microbial growth performance.

These capabilities directly support the aims of this study, which sought to develop a cost-effective and enzyme-based strategy for disrupting the yeast cell wall of *R. toruloides* to enable lipid recovery — a process central to the organism's application in biofuel and oleochemical production (Zhao et al., 2021; Xue et al., 2024).

The findings align with the overarching goals outlined in the thesis titled *"Cell wall disruption by microbial enzymes from soil isolates for efficient downstream processing of Rhodotorula toruloides for lipid production"*. In particular, the identification of *S. seoulensis* as a yeast-lytic candidate meets several objectives: (1) isolating and identifying microorganisms with cell wall-degrading activity, (2) confirming microbial identities via 16S rRNA sequencing and BLASTn analysis, and (3) assessing growth on *R. toruloides* cell wall as a sole carbon source. The genome-based discovery of lytic enzymes also paves the way for future evaluation of enzyme localization and function, aiding in the selection of optimal biocatalysts for lipid extraction workflows. Given that *R. toruloides* is a promising industrial yeast due to its high lipid content and favorable fatty acid profile (Bonturi et al., 2022; Madigan et al., 2020), these results contribute meaningfully to developing enzymatic pre-treatment methods that enhance downstream lipid processing.

These findings are consistent with prior biochemical studies showing that synergistic action of multiple enzyme classes—such as glucanases, chitinases, and proteases—is key to effective yeast cell wall breakdown (Ballesta, 1972). Similar

enzymatic strategies have also been reported in other yeast-lytic microbes, including *Cellulosimicrobium cellulans* and *Bacillus circulans*, which rely on glucanases and mannanases for hydrolyzing yeast wall components (Tanaka & Phaff, 1965; Shibasaki et al., 2008).

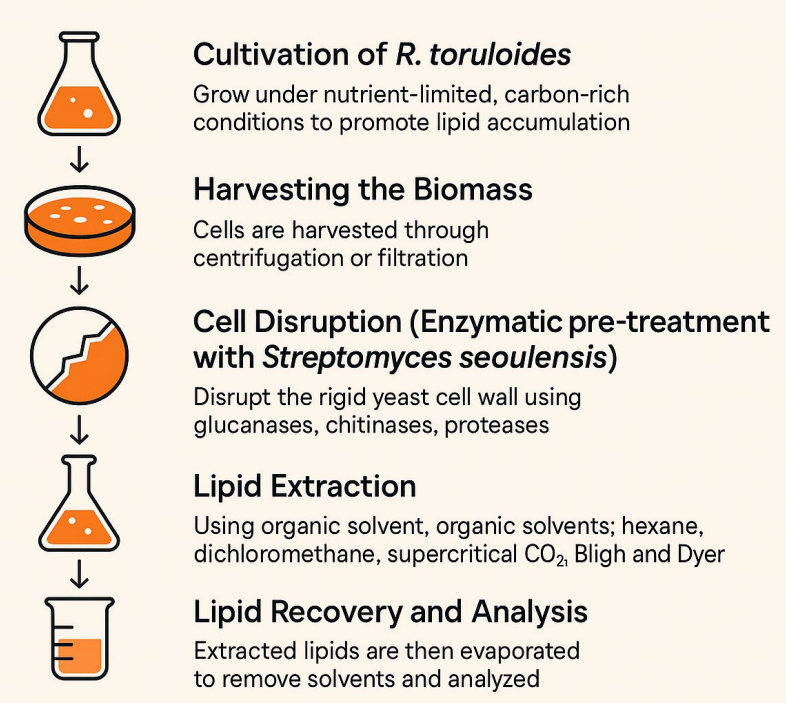

Figure 4. Lipid Production and Extraction Workflow from *Rhodotorula toruloides.*

This figure outlines the five key steps involved in microbial oil production from *R. toruloides*. The process begins with yeast cultivation under nitrogen-limited, carbon-rich conditions to induce lipid accumulation. Under these stress conditions, yeast cells shift metabolism toward de novo lipid biosynthesis, converting excess carbon (e.g., glucose, xylose, or lignocellulosic sugars) into intracellular triacylglycerols (TAGs). These lipids are stored in cytoplasmic lipid bodies and can constitute up to 70% of the yeast's dry cell weight. One of its greatest advantages lies in its ability to utilize a wide variety of low-cost and industrial waste substrates, which significantly reduces the cost of microbial oil production and enhances environmental sustainability. It efficiently utilizes lignocellulosic hydrolysates, palm oil mill effluent, waste glycerol, and food industry residues including crude glycerol, sugarcane hydrolysate, corn steep liquor, molasses, fruit-processing waste, lignocellulosic hydrolysates, de-oiled biomass, and starch-rich byproducts such as potato and cassava waste—even in the presence of fermentation inhibitors—while achieving high lipid yields (Xue et al., 2024; Justine et al., 2022; Szotkowski et al., 2023; Zhao et al., 2021). This flexibility allows *R. toruloides* to utilize lignocellulosic hydrolysates and crude glycerol, as demonstrated in our group's recent studies (Passoth et al., 2023; Martín-Hernández et al., 2023), in addition to other substrates cited previously.

After cultivation, the *Rhodotorula toruloides* biomass is harvested by centrifugation or filtration, followed by a cell disruption step. Given the yeast's thick and complex cell wall, mechanical or chemical disruption is often necessary to access intracellular lipids. As an alternative, an enzymatic pre-treatment using *Streptomyces seoulensis* can be introduced.

Following enzymatic disruption lipids can be extracted using organic solvents like hexane, dichloromethane, or chloroform–methanol mixtures (Schneiter & Daum, 2006). Other methods like supercritical CO_2 which do not need organic solvents can also be utilized (Duarte et al., 2017). Additionally, Karlsson et al. (2016) highlighted that drying yeast biomass prior to extraction, or using dry extraction methods, is among the most efficient approaches for maximizing lipid recovery.

Enzymatic Cell Wall Disruption Using *Streptomyces seoulensis*

Efficient extraction of intracellular lipids from *Rhodotorula toruloides* requires effective disruption or degradation of its robust cell wall, which is potentially composed of β-glucans, chitin, mannans, and glycoproteins (Antunes et al., 2024). Traditional methods like bead-beating or acid treatment can be energy-intensive, equipment-heavy, or damaging to the lipid structure. As an alternative, this study highlights *Streptomyces seoulensis* as a potent biological tool for enzymatic cell wall degradation.

According to the genome annotation of *S. seoulensis*, genes encoding β-1,3-glucanases, chitinases, and proteases were identified, targeting structural components of fungal cell walls. Similar cell wall-degrading activities have been reported for other *Streptomyces* species (Tanaka & Phaff, 1965).

In practice, *Streptomyces seoulensis* could be cultivated separately using inactivated *Rhodotorula toruloides* biomass as the carbon source to induce the expression of cell wall-degrading enzymes. The resulting enzyme-rich culture supernatant could then be harvested and applied to *R. toruloides* biomass as a biological pre-treatment step prior to lipid extraction. This indirect enzymatic approach allows for the isolation and controlled application of extracellular enzymes without introducing live bacterial cultures into the yeast fermentation system.
Alternatively, it may also be feasible to apply **live, pure cultures of *S. seoulensis*** directly to *R. toruloides* biomass, especially in post-fermentation processing steps. This would allow for **in situ degradation of the yeast cell wall**, provided the process is carefully controlled to prevent microbial contamination or competition during lipid accumulation. Such co-culture or sequential treatment strategies have been explored with other microbial systems and could offer practical advantages in certain setups (Tanaka & Phaff, 1965).

Regarding the enzymatic mechanisms, the current study relies on **genome-based annotation** to predict the presence of a suite of **cell wall-degrading enzymes** in *S. seoulensis*, including β-1,3-glucanases, chitinases, proteases, and GPI-cleaving enzymes. These annotations suggest that *S. seoulensis* possesses the genetic potential to secrete such enzymes, but **experimental validation—such as enzyme activity assays or proteomic analysis of culture supernatants—is required** to confirm their actual extracellular secretion under fermentation conditions. Genome annotation alone does not definitively prove enzyme secretion or activity, though *Streptomyces* species are well-known for their robust extracellular enzyme production (Tanaka & Phaff, 1965); (Shibasaki et al., 2008).

This strategy enhances lipid accessibility by biologically weakening the cell wall without the drawbacks of mechanical or chemical methods. Enzymatic pre-treatment has also been shown to improve lipid yields in other yeast systems and supports a more sustainable, low-energy bio-refinery model (Wei et al., 2023).

Identification of Other Microorganisms Capable of Yeast Cell Wall Degradation

In addition to *Streptomyces seoulensis*, several other microorganisms are known to produce enzymes capable of degrading yeast cell walls by targeting structural components such as β-glucans, mannans, chitin, and glycoproteins. Literature screening identified the following key microbial species with verified yeast-lytic activity:

Cellulosimicrobium cellulans: This Gram-positive bacterium is widely recognized for its production of β-1,3-glucanases, mannanases, and proteases. These enzymes are commonly applied in biotechnology for lysing yeast cells, facilitating protoplast formation, and assisting in nucleic acid extraction (Ferrer, 2006).

***Oerskovia* species**: Soil-derived bacteria such as *Oerskovia xanthineolytica* secrete extracellular enzymes, including β-1,3-glucanases and proteases, which are capable of lysing both log-phase and stationary-phase yeast cells. These enzymes degrade cell wall polysaccharides and facilitate spheroplast formation (Scott and Schekman, 1980).

Bacillus circulans: This species is known to produce β-1,3- and β-1,6-glucanases with optimal enzymatic activity at neutral pH. These properties make it useful for controlled enzymatic disruption of yeast cell walls (Rombouts and Phaff, 1976).

Candida oleophila: Although a yeast species, *C. oleophila* exhibits antagonistic behavior against other fungi by secreting lytic enzymes such as chitinases, proteases, and β-1,3-glucanases. These activities contribute to its role as a postharvest biocontrol agent (Yike, 2011).

Chaetomium cupreum: This filamentous fungus produces a broad spectrum of cell wall-degrading enzymes, including glucanases and proteases, and has been used in agricultural settings for biocontrol due to its efficacy in suppressing plant pathogens (Lorito et al., 1994).

These findings underscore the diversity of microbial strategies for yeast cell wall degradation and highlight alternative enzyme sources for biotechnological applications such as biomass processing, fungal control and lipid extraction.

Comparison with Related Studies on Yeast Cell Wall Degradation

The results obtained in this thesis are well supported by previous literature investigating microbial interactions with yeast cell wall components. The isolation and growth characterization of *Streptomyces seoulensis* on *Rhodotorula toruloides* cell wall fractions as a sole carbon source parallels the findings of Scott and Schekman (1980), who demonstrated that soil bacteria such as *Oerskovia xanthineolytica* possess yeast-lytic activity through the secretion of extracellular enzymes capable of degrading yeast cell walls. Similarly, the enzymatic profile annotated in *S. seoulensis*, including β-1,3-glucanases and proteases, corresponds with the work of Ballesta (1972), who described the enzymatic lysis of yeast cells through glucan-targeting enzymes, and Ferrer et al. (2006), who characterized cell wall lytic enzymes from *Cellulosimicrobium cellulans*.

Furthermore, the OD_{600}-based growth monitoring and biomass quantification approach employed in this thesis mirrors methodologies used in quantitative assays evaluating microbial binding and utilization of yeast cell wall substrates (Rombouts & Phaff, 1976). While this study focused on *R. toruloides*, the findings reflect broader enzymatic trends across yeast-degrading microbes and reinforce the potential of soil-derived strains in biotechnological applications, particularly for enhancing lipid recovery through enzymatic pre-treatment.

Answers to research questions

1. Which microbial isolates grow fastest and exhibit highest enzymatic activity on *R. toruloides*?

- **Strain 5**, identified as *Streptomyces seoulensis*, showed the highest growth in both OD_{600} absorbance (peaking at 0.78 on day 4) and dry cell weight (increased from 0.0012 g to 0.0298 g).
- **Strain 4**, also a *Streptomyces* species, showed strong growth, with biomass increasing from 0.0026 g to 0.0166 g over 4 days.

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2. How do OD_{600} growth trends correlate with cell wall degradation?

- Growth trends measured by OD_{600} closely matched biomass data, indicating that strains with higher OD_{600} values (like Strain 5 and 4) were likely utilizing yeast cell wall components effectively—implying active enzymatic degradation of the cell wall.

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3. How can microscopy and OD_{600} analysis quantify degradation?

- OD_{600} and dry cell weight were used to **quantify growth** as a proxy for degradation activity. Although microscopy was mentioned, the thesis primarily relied on quantitative spectrophotometric (OD_{600}) and gravimetric (DCW) measurements for comparison across strains.

Future work paragraph

One important open question concerns which specific enzymes contribute most to yeast cell wall degradation. While genome annotation of *S. seoulensis* (Strain 5) indicated the presence of multiple β-glucanases, chitinases, proteases, and GPI-cleaving enzymes, no actual activity assays were performed. Therefore, identifying the contribution of each enzyme class to cell wall degradation remains a key research objective. This could be addressed in future studies using targeted enzyme assays, gene knockouts, or overexpression systems to validate their roles under process-relevant conditions.

Furthermore, future work should investigate whether these enzymes are secreted into the medium or remain cell-associated, since no enzyme localization experiments were performed in this thesis. Another promising direction would be to examine synergistic effects of enzyme combinations from multiple microbial isolates, potentially through co-culture or enzyme cocktails, to achieve more efficient cell wall breakdown. Finally, testing whether enzyme-based pre-treatment of *R. toruloides* biomass improves lipid extraction yields will be essential to confirm the industrial relevance of the proposed approach.

While *Streptomyces seoulensis* has demonstrated robust potential for degrading the yeast cell wall of *Rhodotorula toruloides* genome annotation indicated the absence of catalytic mannanase activity, which is critical for targeting outer mannoproteins of the yeast cell wall. To address this enzymatic gap, future work could focus on engineering *S. seoulensis* to express heterologous mannanase genes, codon-optimized for *Streptomyces* and equipped with efficient secretion signals. The pCRISPomyces system enables high-efficiency CRISPR/Cas9-mediated editing in *Streptomyces* species, achieving up to 100% editing efficiency (Cobb et al., 2014). Alternative tools like CRISPR-BEST provide DSB-free genome editing, and CRISPRi offers multiplex gene regulation, minimizing genomic instability often associated with actinomycete engineering (Tong et al., 2020; Zhao et al., 2018).

In addition to genetic enhancements, experimental validation of predicted enzyme functions remains crucial. Enzyme assays such as DNS for glucanases and chromogenic substrates for proteases and chitinases could be employed to confirm activity under growth conditions relevant to lipid recovery. Furthermore, it would be essential to assess enzyme localization, distinguishing between extracellular secretion and cell-bound forms. Techniques such as Western blotting or zymogram analyses can help determine secretion efficiency, which has significant implications for downstream processing (Madigan et al., 2020).

Optimizing fermentation conditions—including pH, temperature, agitation, and nutrient composition—may also enhance enzymatic expression and microbial growth. These process parameters are known to influence both enzyme yields and lipid recovery efficiency (Kot et al., 2020). Another promising direction involves designing synergistic microbial consortia, where complementary microbes co-degrade the cell wall (Kaul et al., 1993). Co-culturing *Streptomyces seoulensis* with yeast-degrading fungi or other actinomycetes capable of producing mannanase enzymes could compensate for the lack of mannanase genes in the *S. seoulensis* genome, enabling more complete degradation of yeast cell walls and enhancing lipid release from *Rhodotorula toruloides*.

For industrial relevance, future work should include a preliminary techno-economic analysis (TEA) to evaluate the cost-benefit ratio of implementing enzyme-based pretreatments. Such analysis could estimate resource requirements, scalability, and cost per unit of lipid extracted. Finally, validating engineered strains at the bioreactor or pilot scale would confirm scalability and commercial feasibility, which is vital for industrial deployment (Andrews & Asenjo, 1987).

Deciphering *Rhodotorula toruloides* Cell Wall Structure: A Key Future Direction

A critical future research direction will be the detailed structural characterization of the *R. toruloides* cell wall, which is essential for optimizing targeted enzymatic treatments and improving intracellular lipid recovery. The complex and stress-adapted nature of *R. toruloides* cell walls has been shown to contribute to resistance against mechanical and enzymatic disruption (Antunes et al., 2024).

Solid-state NMR spectroscopy offers a powerful method for gaining atomic-level insights into the polysaccharide matrix. Techniques such as ^13C NMR and 2D solid-state NMR can be applied to isolated cell wall samples to reveal:

- Monomer composition (β-glucans, chitin, mannose, and others)
- Linkage types and branching patterns (e.g., β-1,3 vs. β-1,6 glucans)
- Crystalline versus amorphous regions, particularly within chitin
- Interconnectivity and spatial organization of polysaccharides

In addition, high-resolution magic-angle spinning (HR-MAS) NMR can be employed for hydrated samples, preserving native-like architecture during analysis (Kang et al., 2018).

Recent studies on evolved *R. toruloides* strains demonstrated significant shifts in cell wall polysaccharide composition, with increased levels of glucomannans, fucogalactomannans, and chitin contributing to greater mechanical robustness (Antunes et al., 2024).

By applying these advanced NMR-based methods, future research can generate a comprehensive structural model of the *R. toruloides* cell wall. This will inform the

rational design of enzyme cocktails that more effectively disrupt the cell wall, thereby enhancing lipid release for downstream biofuel and oleochemical production.

Strengths and Limitations

Strengths:

- **Dual growth assessment:** Use of both OD_{600} and dry cell weight (DCW) provided cross-validation for microbial growth trends.
- **Confirmed microbial identity:** Strain 5 (*Streptomyces seoulensis*) was reliably identified through 16S rRNA sequencing and BLASTn alignment.
- **Robust genome annotation:** Annotation tools (Prokka, dbCAN2) revealed a wide repertoire of cell wall-degrading enzymes, particularly β-glucanases and chitinases.
- **Relevance to biotechnology:** Results directly support enzymatic strategies for sustainable lipid extraction from *R. toruloides* biomass.
- **Comparative literature support:** Findings aligned with previous studies on yeast-lytic microorganisms, supporting the biological significance of strain 5.

Limitations:

- **Inconsistent media dispersion:** Yeast cell wall fractions were difficult to fully dissolve in the cell wall medium, causing variation in OD_{600} measurements depending on how well the media was shaken before sampling.
- **No mannanases detected:** *S. seoulensis* lacked annotated mannanase genes, limiting full degradation of mannoproteins in the yeast cell wall.
- **Lack of activity validation:** The presence of lytic enzymes was inferred from annotation only—no biochemical assays or protein secretion studies were performed.
- **Limited microbial diversity:** Only a subset of isolates was tested, potentially overlooking strains with superior lytic performance.
- **Lipid extraction not measured:** Although lipid release and recovery is an important long-term objective for valorizing *R. toruloides* biomass, it was not directly tested in this study.

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Popular science summary

As the world searches for sustainable alternatives to fossil fuels, scientists are turning to microbes for help. One such microbe is *Rhodotorula toruloides*, a yeast that naturally produces large amounts of oil—similar to plant oils like rapeseed—which can be used for food, feed, or even biofuels. However, extracting this oil efficiently is a challenge because the yeast has a tough, protective cell wall.

This project explored whether natural microbes from soil could produce enzymes capable of breaking down the yeast's cell wall, making it easier to release the oil. Several soil bacteria and fungi were tested for their ability to grow on yeast cell wall material, and one strain in particular, identified as a Streptomyces sp. closely related to S. seoulensis, showed strong growth and promise. Genetic analysis revealed that it produces several enzymes known to break down complex structures like those found in yeast walls.

The findings suggest that soil microbes like the tested Streptomyces sp., closely related to S. seoulensis, could be used as a natural, cost-effective tool to improve oil extraction from yeasts, making the process more efficient and eco-friendlier. This could be a step forward in developing greener methods for producing bio-based fuels and products.

Appendix 1

Nucleotide sequences used for blast analysis of strain 4

Sequence 1: Final sequence from SD_02 (used in BLAST search) :Strain4_SD02_16S_sequence

ATGCAGTCCGAACGATGAACCACTTTCCGGTGGGGATTAGTGGCGAAC
GGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCT
GGAAACGGGGTCTAATACCGGATAACACTGCGGACTGCATGGTCTGC
GGTTAAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTG
TTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTG
AGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATG
CAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTT
CAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTT
GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCG
ATTGTGAAAGCCCGAGGCTTAACCTCGGGTCTGCAGTCGATACGGGCT
AGCTAGAGTGTGGTAGGGGGAGATCGGAATTCCTGGTGTAGCGGTGA
AATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTG
GGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGAACTAAGGTGTTG
GCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCG
CCTGGGGAGTACGGCCGCAAGGCTAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAG
AACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATGGTGC
CCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCCT
GTCCTGA

Figure 4. BLASTn search results showing the top hits for the 16S rRNA gene sequence of the isolate SD_02 (strain 4).

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Streptomyces sp. G6-13 16S ribosomal RNA gene, partial sequence	Streptomyces sp. G6-13	1790	1790	100%	0.0	99.00%	1405	JN866756.1
Streptomyces recifensis strain TU22 16S ribosomal RNA gene, partial sequence	Streptomyces recifensis	1790	1790	100%	0.0	99.00%	1425	MH482880.1
Streptomyces sp. strain XKC-SO-4 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1790	1790	100%	0.0	99.00%	1456	OQ449531.1
Streptomyces sp. strain XKC-SO-16 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1790	1790	100%	0.0	99.00%	1465	OQ449539.1
Streptomyces sp. strain LK-R-7 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1790	1790	100%	0.0	99.00%	1462	OQ456524.1
Streptomyces sp. strain BS-3 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1788	1788	100%	0.0	99.00%	1456	OQ438813.1
Streptomyces sp. NBC_01171 chromosome, complete genome	Streptomyces sp. NBC_0…	1784	10709	100%	0.0	98.90%	6584968	CP108609.1
Streptomyces sp. NBC_01601 chromosome, complete genome	Streptomyces sp. NBC_0…	1784	10709	100%	0.0	98.90%	6514704	CP109314.1
Streptomyces sp. 6G-4 16S ribosomal RNA gene, partial sequence	Streptomyces sp. 6G-4	1784	1784	100%	0.0	98.90%	1408	JN866762.1
Streptomyces sp. NBC_00566 chromosome, complete genome	Streptomyces sp. NBC_0…	1784	10709	100%	0.0	98.90%	6411420	CP107784.1
Streptomyces seoulensis HEK131 DNA, complete genome	Streptomyces seoulensis	1784	10704	100%	0.0	98.90%	6542274	AP025667.1
Streptomyces seoulensis strain HBUM173318 16S ribosomal RNA gene, partial sequence	Streptomyces seoulensis	1784	1784	100%	0.0	98.90%	1449	EU841610.1

Sequence 2: Final sequence from SD_04 (used in BLAST search)

:Strain4_SD04_sequence
CCGGCAGTCTCCTGTGAGTCCCCATCACCCCGAAGGGCATGCTGGCAA
CACAGAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCAC
GACACGAGCTGACGACAGCCATGCACCACCTGTACACCGACCACAAG
GGGGGCACCATCTCTGATGCTTTCCGGTGTATGTCAAGCCTTGGTAAG
GTTCTTCGCGTTGCGTCGAATTAAGCCACATGCTCCGCTGCTTGTCGCG
GGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAG
GCGGGGAACTTAATGCGTTAGCTGCGGCACCGACGACGTGGAATGTC
GCCAACACCTAGTTCCCACCGTTTACGGCGTGGACTACCAGGGTATCT
AATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAATGGCCC
AGAGATCCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCA
CCGCTACACCAGGAATTCCGATCTCCCCTACCACACTCTAGCTAGCCC
GTATCGACTGCAGACCCGAGGTTAAGCCTCGGGCTTTCACAATCGACG
TGACAAGCCGCCTACGAGCTCTTTACGCCCAATAATTCCGGACAACGC
TTGCGCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGC
TTCTTCTGCAGGTACCGTCACTTTCGCTTCTTCCCTGCTGAAAGAGGTT
TACAACCCGAAGGCCGTCATCCCTC

Figure 5. BLASTn search results showing the top hits for the 16S rRNA gene sequence of the isolate SD_04 (strain 4).

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Streptomyces sp. NBC_01171 chromosome, complete genome	Streptomyces sp. NBC_01171	1367	8205	100%	0.0	99.87%	6584968	CP108609.1
☑	Streptomyces sp. LaPpAH181 16S ribosomal RNA gene, partial sequence	Streptomyces sp. LaPpAH181	1367	1367	100%	0.0	99.87%	1375	KJ889046.1
☑	Streptomyces sp. strain 4213b 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1367	1367	100%	0.0	99.87%	1252	KX573788.1
☑	Streptomyces seoulensis strain AB770 16S ribosomal RNA gene, partial sequence	Streptomyces seoulensis	1367	1367	100%	0.0	99.87%	1119	MN326857.1
☑	Streptomyces sp. UT1123 16S ribosomal RNA gene, partial sequence	Streptomyces sp. UT1123	1367	1367	100%	0.0	99.87%	1362	KT354639.1
☑	Streptomyces sp. NBC_01601 chromosome, complete genome	Streptomyces sp. NBC_01601	1367	8200	100%	0.0	99.87%	6514704	CP109314.1
☑	Streptomyces sp. strain RB107 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1367	1367	100%	0.0	99.87%	1373	KY558691.1
☑	Streptomyces sp. G6-13 16S ribosomal RNA gene, partial sequence	Streptomyces sp. G6-13	1367	1367	100%	0.0	99.87%	1405	JN866756.1
☑	Streptomyces sp. NBC_00566 chromosome, complete genome	Streptomyces sp. NBC_00566	1367	8205	100%	0.0	99.87%	6411420	CP107784.1
☑	Streptomyces recifensis strain TU22 16S ribosomal RNA gene, partial sequence	Streptomyces recifensis	1367	1367	100%	0.0	99.87%	1425	MH482880.1
☑	Streptomyces sp. strain PR181 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1367	1367	100%	0.0	99.87%	1409	MZ393616.1
☑	Actinobacterium R42 16S ribosomal RNA gene, partial sequence	actinobacterium R42	1367	1367	100%	0.0	99.87%	1443	KP292585.1

The query sequence showed 100% coverage and 99.60% identity with multiple *Streptomyces seoulensis* strains as well as an unclassified *Streptomyces sp.* XFole15. The results were obtained using NCBI BLASTn (accessed on [2025-04-02]

Nucleotide sequences used for blast analysis of strain 5

Sequence 1: Final sequence from SD_01 (used in BLAST search)

Strain 5_SD_01:
CAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTG
AGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAA
CGGGGTCTAATACCGGATAACACTGCGGACTGCATGGTCTGCGGTTAA

AAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTG
AGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGC
GACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGAC
GCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGG
GAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAA
TTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGATTGTGAA
AGCCCGAGGCTTAACCTCGGGTCTGCAGTCGATACGGGCTAGCTAGAG
TGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAG
ATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTAC
TGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGGTGGGAACTAAGGTGTTGGCGACATTC
CACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCCGGGGGA
GTACGGCCGCAAGGCTAAAACTCAAAGGAATTTGACGGGGGCCCGCA
CAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTT
ACCAAGGCTTGACATACACCGGAAAGCATCC

Figure 6. BLASTn search results showing the top hits for the 16S rRNA gene sequence of the isolate SD_01 (strain 5).

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Streptomyces recifensis strain LZ13-16 16S ribosomal RNA gene, partial sequence	Streptomyces recifensis	1683	1683	100%	0.0	99.36%	1430	MT856247.1
Streptomyces sp. G6-13 16S ribosomal RNA gene, partial sequence	Streptomyces sp. G6-13	1683	1683	100%	0.0	99.36%	1405	JN866756.1
Streptomyces recifensis strain TU22 16S ribosomal RNA gene, partial sequence	Streptomyces recifensis	1683	1683	100%	0.0	99.36%	1425	MH482880.1
Streptomyces sp. strain BS-3 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1683	1683	100%	0.0	99.36%	1456	OQ438813.1
Streptomyces sp. strain XKC-SO-4 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1683	1683	100%	0.0	99.36%	1456	OQ449531.1
Streptomyces sp. strain XKC-SO-16 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1683	1683	100%	0.0	99.36%	1465	OQ449539.1
Streptomyces sp. strain LK-R-7 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1683	1683	100%	0.0	99.36%	1462	OQ456524.1
Streptomyces sp. NBC_01171 chromosome, complete genome	Streptomyces sp. NBC_01171	1681	10089	100%	0.0	99.35%	6584968	CP108609.1
Streptomyces sp. NBC_01601 chromosome, complete genome	Streptomyces sp. NBC_01601	1681	10089	100%	0.0	99.35%	6514704	CP109314.1
Streptomyces sp. strain A1314 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1681	1681	100%	0.0	99.35%	1010	KU865524.1
Streptomyces sp. strain A1303 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1681	1681	100%	0.0	99.35%	1011	KU865516.1
Streptomyces sp. NBC_00566 chromosome, complete genome	Streptomyces sp. NBC_00566	1681	10089	100%	0.0	99.35%	6411420	CP107784.1
Streptomyces sp. 6IS-1 16S ribosomal RNA gene, partial sequence	Streptomyces sp. 6IS-1	1681	1681	100%	0.0	99.35%	1402	JN866761.1
Streptomyces seoulensis HEK131 DNA, complete genome	Streptomyces seoulensis	1681	10083	100%	0.0	99.35%	6542274	AP025667.1
Streptomyces seoulensis strain HBUM173318 16S ribosomal RNA gene, partial sequence	Streptomyces seoulensis	1681	1681	100%	0.0	99.35%	1449	EU841610.1

Sequence 2: Final sequence from SD_03 (used in BLAST search)

Strain 5_SD_03:

CCGGCAGTCTCCTGTGAGTCCCCATCACCCCGAAGGGCATGCTGGCAA
CACAGAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCAC
GACACGAGCTGACGACAGCCATGCACCACCTGTACACCGACCACAAG
GGGGGCACCATCTCTGATGCTTTCCGGTGTATGTCAAGCCTTGGTAAG

GTTCTTCGCGTTGCGTCGAATTAAGCCACATGCTCCGCTGCTTGTGCGG
GCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGG
CGGGGAACTTAATGCGTTAGCTGCGGCACCGACGACGTGGAATGTCGC
CAACACCTAGTTCCCACCGTTTACGGCGTGGACTACCAGGGTATCTAA
TCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAATGGCCCAG
AGATCCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACC
GCTACACCAGGAATTCCGATCTCCCCTACCACACTCTAGCTAGCCCGT
ATCGACTGCAGACCCGAGGTTAAGCCTCGGGCTTTCACAATCGACGTG
ACAAGCCGCCTACGAGCTCTTTACGCCCAATAATTCCGGACAACGCTT
GCGCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTT
CTTCTGCAGGTACCGTCACTTTCGCTTCTTCCCTGCTGAAAAGAGGTTT
ACAACCCGAAGGCCGTCATC

Figure 7. BLASTn search results showing the top hits for the 16S rRNA gene sequence of the isolate SD_03 (strain 5).

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Streptomyces sp. NBC_01171 chromosome, complete genome	Streptomyces sp. NBC_01171	1367	8205	100%	0.0	99.87%	6584968	CP108609.1
Streptomyces sp. LaPpAH181 16S ribosomal RNA gene, partial sequence	Streptomyces sp. LaPpAH181	1367	1367	100%	0.0	99.87%	1375	KJ889046.1
Streptomyces sp. strain 4213b 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1367	1367	100%	0.0	99.87%	1252	KX573788.1
Streptomyces seoulensis strain AB770 16S ribosomal RNA gene, partial sequence	Streptomyces seoulensis	1367	1367	100%	0.0	99.87%	1119	MN326857.1
Streptomyces sp. UT1123 16S ribosomal RNA gene, partial sequence	Streptomyces sp. UT1123	1367	1367	100%	0.0	99.87%	1362	KT354639.1
Streptomyces sp. NBC_01601 chromosome, complete genome	Streptomyces sp. NBC_01601	1367	8200	100%	0.0	99.87%	6514704	CP109314.1
Streptomyces sp. strain RB107 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1367	1367	100%	0.0	99.87%	1373	KY558691.1
Streptomyces sp. G6-13 16S ribosomal RNA gene, partial sequence	Streptomyces sp. G6-13	1367	1367	100%	0.0	99.87%	1405	JN866756.1
Streptomyces sp. NBC_00566 chromosome, complete genome	Streptomyces sp. NBC_00566	1367	8205	100%	0.0	99.87%	6411420	CP107784.1
Streptomyces recifensis strain TU22 16S ribosomal RNA gene, partial sequence	Streptomyces recifensis	1367	1367	100%	0.0	99.87%	1425	MH482880.1
Streptomyces sp. strain PR181 16S ribosomal RNA gene, partial sequence	Streptomyces sp.	1367	1367	100%	0.0	99.87%	1409	MZ393616.1

Biomass experiment calculations

Strain 4

Flask id	Time point	Tube weight (g)	Tube + biomass (g)	Dry cell weight (g)
1	0	12,5396	12,5422	0,0026
4	4	12,5335	12,5506	0,0171
5	4	12,7518	12,7726	0,0208
6	4	12,5383	12,5502	0,0119

Time points	Mean DCW (g)
0	0,0026
4	0,0166

C1V1 = C2V2

V2 = 20 ml
C2 = 0.05
C1 = 0.87
V1 = ?

V1 = C2 x V2 / C1

0.05 x 20 / 0.87 = 1.15 ml

20 – 1.15 = 18.85 ml

For strain 4, biomass experiment 2, 18.85 ml of cell wall media and 1,15 ml of pre-culture were added to six 125 ml bottles.

Calculations regarding strain 5

Strain 5
C1 = 1,01
V1 = (0,05 x 20)/ 1,01
V1 = 0,99 ml
20 - 0,99 = 19,01 ml

Time point	Dry cell weight DCW (g)
0	0,0071
0	0,0176
4	0,0296
4	0,03

Time point	Mean DCW (g)
0	0,01235
4	0,0298

Strain 11

	Calculations to get the same initial starting concentration for biomass experiments			
	Strain 11			C1 = OD600 of pre-culture
Preculture	OD600	C2= 0,05	V2= 20 ml	V1 = Volume needed of preculture for inoculatio
	C1= 0,14			C2 = Target OD600
	C1V1=C2V2			V2= Final Volume of growth culture

V1= (C2V2)/C1
(0,05 x 20)/0,14
V1 = 7,14 ml
20 - 7,14 = 12,86ml
To low OD 600 values to proceed with bioexperiment
Low OD values indicate bad growth!

Strain 3:

Strain 3
Blank 0,34
C1 = 0,19
V1 =(0,05 x 20) / 0,19
V1 = 5,26 ml
20 - 5,26 = 14,74 ml

To low OD values to perform biomass experiment regarding strain 3.

Strain 7
C1 = 0,57 Blank 0,17
V1 = (C2 x V2)/C1
V1 = (0,05 x 20) / 0,57
V1 =1,75 ml of preculture
20 - 1,75 = 18,25 ml of cell wall media

Regarding strain 6 and 9

Blank = 0,38

Strain 6 = OD_{600} = 0,00

Strain 9 = OD_{600} = 0,00

To low OD values to perform biomass experiment regarding strain 6 and 9.

Liquid Culture Optical Density (OD_{600}) Measurement calculations

Strain 3 C2 = 0,03
C1 = 0,03 V2 = 50 ml

C1 = OD600 after 1 c
V1 = (C2 x V2) / C1 V1 = amount of volun

V1 = (0,3 x 50) / 0,09
V1 = 16,6 ml
50 - 16,6 = 33,4 ml

Strain 8

C1 = 0,23
V1 = (0,03 x 50)
/0,23
V1 = 6,5 ml
50-6,5 = 43,5 ml

Strain 4
C1 = 1,14
V1 =(0,03 x 50) /
1,14
V1 = 1,31
50 - 1,31 = 48,7
ml

Strain 7
C1 = 0,13
V1 = (0,03 x 50)/0,13
V1 = 11,5 ml
50 - 11,5 = 38,5 ml
Strain 5
C1 = 0,11
V1 = (0,05 x 50) /0,11
V1 = 22,7
50 - 22,7 = 27,3 ml

Strain 11
C1 = 0,42
V1 = (0,05 x 50)
/0,42
V1 = 5,95 ml
50 - 5,95 = 44,05 ml

Strain 9
C1 = 0,14
V1 = 0,01 x 50 /
0,14
V1 = 3,57 ml
50 - 3,57 =
46,43 ml

Strain 6
C1 = 0,05
V1 = (0,01 x 50) /
0,05
V1 = 10 ml
50 - 10 = 40 ml

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