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L(+)-LACTIC ACID PRODUCTION FROM CORNCOB BIOMASS USING MULTIPLE PARALLEL FERMENTATION (MPF) WITH *ASPERGILLUS* SPP. AND *LACTOCOCCUS LACTIS*

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Abstract

Corncob is an abundant lignocellulosic residue whose conversion to high-value chemicals remains challenging. Here, we report the first Multiple Parallel Fermentation (MPF) that couples *Aspergillus niger* ATCC 16888, *Aspergillus flavus* ATCC 26946 and *Lactococcus lactis* ATCC 19435 in one pot for L-(+)-lactic acid production. The fungi supplied complementary cellulase- and xylanase-rich enzyme suites, while LAB rapidly fermented the released sugars. Enzyme activities, consortium compatibility and fermentation conditions were systematically optimized through Taguchi design (temperature 35–39 °C, pH 6.0–7.0, substrate 3–7%). The optimum setting—39 °C, pH 6.0, 7% corncob—yielded 1.783% w/v optically pure L-lactate in 48 h, outperforming earlier corncob processes by 19–55%. ANOVA assigned ~81% of the variance to temperature, confirming its dominant role, whereas substrate concentration and pH accounted for 14% and 4%, respectively. This is the first dual-fungus MPF on corncob. The dual-fungus strategy thus maintains a steady monosaccharide pool that sustains rapid LAB metabolism. Single-step MPF shortens processing time, avoids chemical hydrolysis and valorizes agricultural waste, offering a scalable route to food-grade L-lactic acid and PLA precursors within a circular-bioeconomy framework.

Keywords: *Aspergillus flavus, Aspergillus niger,* Corncob biomass; L(+)-Lactic acid*, Lactococcus lactis,* Multiple Parallel Fermentation, Polysaccharide-Lytic Enzymes

INTRODUCTION

Lactic acid finds extensive use in food, pharmaceutical, and biodegradable plastic industries, including as an additive and biopolymer precursor (Komesu *et al.*, 2017). Demand continues to grow due to increasing consumer awareness of healthier additives and the pursuit of sustainable materials. However, local lactic acid production often relies on chemical synthesis, which yields racemic mixtures. By contrast, microbial fermentation is more eco-friendly and can generate the L(+)-isomer, preferred for its compatibility with human metabolism (Huang *et al.*, 2021).

Corncob biomass, rich in hemicellulose, requires pretreatment to disrupt its cellulose–hemicellulose–lignin structure (Kumar and Sharma, 2017; Baptista *et al.*, 2020). Recent moves away from chemical pretreatments reduce energy consumption and environmental harm (Fernandes Antunes *et al.*, 2019). Multiple Parallel Fermentation (MPF) leverages fungi and lactic acid bacteria (LAB) together, enhancing sugar release and boosting lactic acid production (Mufidah, A. A. Prihanto and Wakayama, 2017; Du *et al.*, 2023). This synergy is crucial to turning agricultural waste into valuable bioproducts (Baptista *et al.*, 2020). Nevertheless, producing L(+)-lactic acid from corncobs faces bottlenecks: lignin obstructs hydrolysis, and limited enzymatic activity restricts sugar availability (Zhang *et al.*, 2019). Single-step methods like simultaneous saccharification and fermentation (SSF) can be hard to control, given the differing optima of enzymes and microbes. Multi-stage systems mitigate these issues but risk contamination. Another approach uses carefully chosen consortia, though success hinges on selecting mutually compatible microbes and optimizing temperature, pH, and substrate levels (Mufidah, Prihanto and Wakayama, 2017).

MPF addresses such challenges by running fungi and LAB together. Fungi produce diverse Polysaccharide-Lytic Enzymes (PLE)—including pectinase, cellulase, and xylanase—to deconstruct corncob, while LAB convert the resulting sugars into L(+)-lactic acid (Mufidah and Wakayama, 2016; Mufidah, A. A. Prihanto and Wakayama, 2017; Li *et al.*, 2023). This parallel configuration reduces fermentation time and maintains robust enzyme activity, minimizing delays between sugar release and utilization (Mufidah, Prihanto and Wakayama, 2017). Prior research has documented improved yields when combining fungal pretreatment with lactic acid fermentation. For instance, Mufidah *et al.* (2017) reported high-purity L(+)-lactic acid from banana peel using MPF. Similar success could extend to corncobs, which are rich in hemicellulose.

However, studies of MPF with corncob substrates specifically remain limited, often focusing on separate hydrolysis or less integrated methods. Yue and Zhang (2023) emphasised that fungal–bacterial synergy is vital for efficient conversion, yet optimising factors such as substrate concentration, pH, and temperature for microbial in a single MPF system is still underexplored. This gap highlights the need for further experimentation to fine-tune fermentation parameters.

To the best of our knowledge, this study provides the first demonstration of a dual-fungus system—operated in Multiple Parallel Fermentation (MPF) on corncob. The consortium delivered optically-pure L(+)-lactic acid. These results highlight the benefit of pairing the complementary cellulase-rich profile of *Aspergillus niger* with the xylanase dominance of *Aspergillus flavus*, thereby maintaining a balanced sugar pool for *Lactococcus lactis* fermentation.

Accordingly, this research investigates L-(+)-lactic acid production via MPF from fungi for enzymatic hydrolysis with lactic acid bacteria for fermentation, using pre-treated corncob (70 g L^{-1}) as the sole substrate. We hypothesise that such a consortium will deliver higher L(+)-lactic acid titres than conventional or single-step approaches. Employing a Taguchi L9 design, we systematically optimise pH, temperature and substrate loading while profiling microbial synergy and enzyme activities. To the best of our knowledge, this is the first report of a dual-fungus MPF that converts corncob directly into optically pure L-lactic acid, thereby expanding the toolbox for lignocellulose valorisation.

MATERIALS AND METHODS

Materials

A variety of materials supported microbial growth and enzymatic assays. Distilled water and calcium carbonate ($CaCO_3$; Merck, Germany) were used for media preparation and pH regulation. MRS agar/broth (HiMedia, India) was used to cultivate lactic acid bacteria, while fungi were grown on Potato Dextrose Broth or Potato Dextrose Agar (PDB or PDA) with malt extract (Oxoid, UK). Tannic acid (Sigma-Aldrich, USA) indicated enzyme activity via brown coloration. Additional components such as polypeptone (Difco, USA), beef extract (Difco, USA), yeast extract (Oxoid, UK), glucose (Merck, Germany), and buffers including sodium acetate (Sigma-Aldrich, USA) and dipotassium hydrogen phosphate (Merck, Germany), along with diammonium hydrogen citrate (Merck, Germany), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$; Sigma-Aldrich, USA), and manganese(II) sulfate monohydrate ($MnSO_4{\cdot}H_2O$; Sigma-Aldrich, USA), supported microbial growth and fermentation. Substrates used for polysaccharide-lytic enzyme assays—pectin, xylan, carboxymethyl cellulose (CMC), salicin, and starch—were obtained from Sigma-Aldrich (USA), and reducing sugars were quantified using the DNS method (3,5-dinitrosalicylic acid; Sigma-Aldrich, USA). Corn cob biomass pretreatment was conducted using dilute sulfuric acid (0.1% H_2SO_4) at 60 °C for 12 hours, with a solid-to-liquid ratio of 1:10 (w/v), followed by neutralization, filtration, and autoclaving at 120 °C for 1 hour before microbial inoculation. For lactic acid analysis, reagents including glycylglycine buffer, L-glutamic acid, NAD^+, and lactate dehydrogenase (all from R-Biopharm, Germany) were used for spectrophotometric detection of NADH.

Instruments

Several instruments were used to support the experiments and data analysis. A Shimadzu UV-Vis Spectrophotometer (UV-1800, Shimadzu Corporation, Japan) measured optical density for bacterial growth and enzyme activity. Microscopic

observations were carried out using an Olympus CX23 Microscope (Olympus Corporation, Japan). An Eppendorf 5430R Refrigerated Centrifuge (Eppendorf, Germany) was used to separate biomass from supernatants. Sterile conditions were maintained using a Biobase BBS-V800 Laminar Airflow Cabinet (Biobase, China). Microbial cultivation was conducted in a Memmert Incubator (Memmert GmbH, Germany). Data analysis and visualization were performed using Microsoft Excel (Microsoft Corporation, USA) and SPSS Statistics software (IBM, USA).

Preparation of Microbial Cultures

Growth Curve Observation of Lactic Acid Bacteria (LAB)

An initial starter culture was prepared by inoculating LAB (*Lactococcus lactis* ATCC 19435, *Lactobacillus bulgaricus* ATCC 11842 and *Leuconostoc mesenteroides* ATCC 8293) isolates into 130 mL of MRS B containing $CaCO_3$. Each isolate was derived from rejuvenated stocks using five inoculation loops (ose). The cultures were incubated at 37 °C in a shaker incubator to facilitate uniform mixing and aeration. After sufficient growth, 12.25 mL of the resulting culture was transferred to 122.25 mL of fresh MRS B + $CaCO_3$. Samples were taken over 48 hours at 16 distinct time points, with each time point sampled in duplicate. Growth was monitored by measuring the optical density (OD) at 600 nm using the Shimadzu UV-Vis Spectrophotometer, with sterile MRS B as the blank reference. LAB inoculum was prepared by cultivating cells in MRS broth until achieving an optical density (OD_{600}) of approximately 0.8, corresponding to the exponential growth phase.

Qualitative Screening of Ligninolytic Enzymes in Fungi

Fungal isolates (*Aspergillus niger* ATCC 16888, *A. flavus* ATCC 26946, *Rhizopus oryzae* ATCC 56536) were qualitatively screened for ligninolytic enzyme activity through the Bavendamm test, in which fungal mycelia were cultivated on PDA containing 0.5% tannic acid and 1.5% malt extract. Plates were incubated in sealed containers (dark conditions) at 37 °C for 3–5 days. A positive result, indicated by a brown precipitate surrounding the fungal colony, confirmed ligninolytic activity via tannic acid oxidation. This color change is associated with phenol oxidase enzymes characteristic of white-rot fungi.

Polysaccharide-Lytic Enzyme Activity Assays

Preparation of Substrate-Induced Crude Enzyme

Fresh fungal plugs (Ø 6 mm) from actively growing PDA were transferred aseptically to 30 mL sterile PDB. A parallel set of flasks was supplemented with 1% w/v citrus pectin, birch-wood xylan, soluble starch, CMC or salicin to induce the matching hydrolases. After 7 d at 37 °C the broths were filtered and centrifuged (10 000 rpm, 15 min, 4 °C); the clarified supernatant was taken as crude enzyme. The DNS reagent was freshly prepared (modified Miller 1959): dissolve 1.66 g NaOH in 40 mL warm distilled water; add 1.66 g 3,5-dinitrosalicylic acid, stir until clear; add 30.33 g Na-K-tartrate slowly while stirring; continue on magnetic stirrer 2 h for complete dissolution. Add 0.33 g phenol and 0.083 g $NaHSO_3$, make up to 100 mL, filter, store in amber bottle at 4 °C. Sodium-citrate buffer 0.05 M, pH 4.4 was prepared as described and sterile-filtered.

Pectinase Activity Assay

The reaction was assembled by mixing 0.5 mL crude enzyme, 0.25 mL citrus pectin and 0.25 mL sodium-citrate buffer in a test tube. The mixture was incubated for 30 min at room temperature, after which 3 mL DNS reagent was added and the tube was heated in a boiling-water bath (100 °C) for 15 min. Once cooled, a blank containing 0.5 mL distilled water, 0.5 mL citrate buffer plus citrus pectin and 3 mL DNS was prepared. The absorbance of every sample and blank was read at 540 nm with a UV-Vis spectrophotometer. One unit (U) corresponded to 1 µmol galacturonic acid released per minute.

Xylanase Activity Assay

One millilitre of crude enzyme was combined with 2 mL of 1% xylan prepared in phosphate buffer (pH 7). After a 10 min incubation at 60 °C, 2 mL DNS was added and the mixture was boiled for 5–10 min. Following cooling, a blank (1 mL distilled water, 1 mL 1% xylan, 2 mL DNS) was measured at 540 nm, and the optical density (OD) of each sample was recorded at the same wavelength. Absorbance was measured at 540 nm; activity was expressed as U mL^{-1} xylose equivalents.

Endoglucanase Activity Assay

A test tube received 1 mL crude enzyme, 1 mL 1% CMC and 1 mL sodium-citrate buffer (pH 4.8). The mixture was incubated for 30 min at room temperature, then 1 mL DNS was added and the tube was boiled 5–10 min. After cooling, the corresponding blank (1 mL distilled water, 1 mL 1% CMC, 2 mL DNS) was read at 540 nm, and the OD of each sample was determined in the same way.

β-Glucosidase activity assay

One millilitre of crude enzyme, 1 mL citrate buffer (pH 4.8) and 2 mL 1 % salicin were mixed and incubated for 30 min at 50 °C. Two millilitres DNS was then added; the tube was boiled for 5–10 min and allowed to cool. A blank composed of 1 mL distilled water, 1 mL 1% salicin and 2 mL DNS was used for reference, and all absorbances were recorded at 540 nm with a UV-Vis spectrophotometer.

Amylase Activity Assay

The reaction mixture contained 1 mL crude enzyme and 1 mL 1% soluble starch prepared in phosphate buffer (pH 7). After 30 min at room temperature, 2 mL DNS reagent was added and the tube was heated at 100 °C for 5–10 min. After cooling, a blank of 1 mL distilled water, 1 mL 1% starch and 2 mL DNS was prepared, and the OD of blanks and samples was measured at 540 nm.

Microbial Consortium Assay

For each consortium assay, the LAB inoculum—*Lactococcus lactis* ATCC 19435, *Lactobacillus bulgaricus* ATCC 11842 and *Leuconostoc mesenteroides* ATCC 8293—was prepared from overnight cultures in MRS broth (37 °C, 150 rpm). Cells were harvested (5 000 × g, 10 min), washed twice with 0.85% NaCl and resuspended to $OD_{600} \approx 0.8$ ($\approx 10^8$ CFU mL^{-1}). The fungal inoculum—*Aspergillus niger* ATCC 16888, *A. flavus* ATCC 26946 and *Rhizopus oryzae* ATCC 56536—consisted of fresh 6 mm agar plugs cut from the actively growing margin of 3-day PDA cultures. Roughly 20 mL of dual-culture agar medium (1.5% polypeptone, 0.5% yeast extract, 1% beef extract, 2% glucose, 0.7% sodium acetate, 0.3% diammonium hydrogen citrate, 0.4% dipotassium hydrogen phosphate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.004% $MnSO_4 \cdot H_2O$, 0.1% NaCl, 0.2% ammonium sulfate, 0.1% Tween 80, 0.005% $FeSO_4 \cdot 7H_2O$, 0.48 g $CaCO_3$ and 3.12 g PDA per 80 mL water was poured into 90 mm Petri dishes and allowed to solidify. One fungal plug was placed in the centre of each plate, and each LAB suspension was streaked as a straight line along the left and right margins, approximately 20 mm from the plug. Every LAB–fungus combination was prepared in triplicate. Plates were sealed with Parafilm, incubated at 37 °C for 24 h, and inspected qualitatively for colony overlap or clear inhibition at the interface between the LAB streaks and the expanding fungal mycelium. Isolate pairs showing overlap (no inhibition band) were deemed compatible, whereas the presence of an inhibition zone signified antagonism.

Multiple Parallel Fermentation (MPF)

Multiple Parallel Fermentation (MPF) combines fungal hydrolysis and bacterial fermentation in a single system to maximize substrate utilization and lactic acid production. The medium, containing 3% pretreated corncob biomass and essential nutrients, was adjusted to pH 5.6. Fungal inocula (10 mL) were added to 200 mL of medium and incubated at 30 °C for 24 hours with shaking to initiate substrate breakdown. *Lactococcus lactis* was then introduced, and fermentation continued at 37 °C. This simultaneous approach shortens processing time and improves lactic acid yield by converting complex lignocellulosic biomass into valuable products in a more efficient and sustainable manner.

Lactic Acid Quantification

Accurate determination of lactic acid was essential for evaluating fermentation performance. Two methods were employed to measure lactic acid concentration in culture supernatants.

Alkalimetric Titration

An alkalimetric titration procedure was conducted using a standardized NaOH solution. One milliliter of fermented supernatant was diluted to 10 mL in a 250 mL Erlenmeyer flask, then mixed with three drops of 1% phenolphthalein. Lactic acid concentration was determined using:

$$\text{Lactic Acid (\%)} = \frac{V_1 \times N \times EW \times DF \times 100\%}{V_2 \times 1\,000}$$

V_1.....Volume of standardized 0.1131 N NaOH (mL);
N.....Normality of the standardized NaOH solution;
EW...Equivalent weight of lactic acid (90.08 g/eq);
DF.....Dilution factor;
V_2.....Volume of the titrated sample (mL).

Boehringer Mannheim/R-Biopharm

L(+)-lactic acid was also quantified according to the Boehringer Mannheim/R-Biopharm method, with minor modifications. The reaction mixture comprised 222 mM glycylglycine buffer at pH 10.0, 100 mM L-glutamic acid, 52.8 mM NAD^+, 13.66 U of glutamate-pyruvate transaminase, and 43.48 U of L-lactate dehydrogenase. Following the enzymatic conversion of L-lactate into NADH, absorbance was measured at 340 nm to track NADH formation. In some cases, 6.79 U of L-lactate dehydrogenase was substituted to verify consistency in the measurements. This spectrophotometric approach provided a sensitive measure of L-lactic acid concentrations, complementing the titration results and enabling a comprehensive evaluation of lactic acid production.

Research Design

This study optimises L(+)-lactic acid production via Multiple Parallel Fermentation by simultaneously examining pH, temperature, and substrate concentration. Three pH settings (6.0, 6.5, 7.0), temperatures (35, 37, 39 °C) and substrate loads (3, 5, 7% w/v) are arranged within an L9 Taguchi orthogonal array, replacing a full factorial with nine trials. Each run is replicated in a completely randomised design to enhance statistical confidence. Fermentation yield (%) constitutes the primary response, while the signal-to-noise ratio identifies robust conditions by penalising variability. This integrated approach pinpoints the most favourable physicochemical window for microbial activity and enzyme kinetics, accelerating scalable bioprocess development in industry.

RESULTS AND DISCUSSION

Enzyme Activity Measurement

General Overview

Enzyme activity measurements are crucial for understanding the capacity of fungal isolates to break down lignocellulosic materials (in this case, corncobs) into simpler sugars such as glucose and xylose. Fungal species, particularly those belonging to the genus Aspergillus and Rhizopus, produce a diverse range of enzymes—endoglucanase, pectinase, beta-glucosidase, amylase, and xylanase—that target different components of lignocellulosic biomass. By quantifying enzyme activity over time, we can pinpoint the optimal incubation periods that lead to maximal enzymatic degradation, thereby producing fermentable sugars more efficiently.

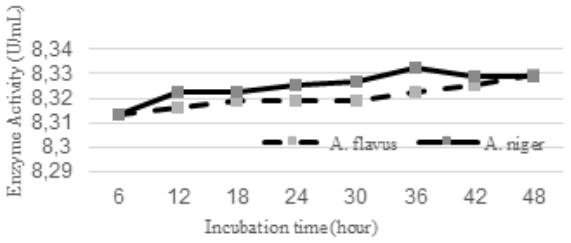
In this study, enzyme activities of *Aspergillus flavus, Aspergillus niger,* and *Rhizopus oryzae* were monitored using the DNS (3,5-dinitrosalicylic acid) method. Crude enzyme extracts were collected every 48 hours, and the reduction of DNS was used to quantify the release of reducing sugars from specific substrates (e.g., carboxymethyl cellulose for endoglucanase, pectin for pectinase, etc.). The resulting absorbance at 540 nm was converted to enzyme units (U/mL) using standard curves of known concentrations of simple sugars.

Endoglucanase Enzyme

Endoglucanases are integral to the hydrolysis of cellulose by cleaving internal β-1,4-glycosidic bonds within cellulose chains. The activity of endoglucanase in *Aspergillus niger ATCC 16888* and *Aspergillus flavus ATCC 26946* was measured every 48 hours Fig. 1. Both isolates exhibited increasing enzyme activity over the incubation period, signifying progressive enzyme production.

A paired t-test was performed to evaluate the difference in endoglucanase activity between *Aspergillus niger ATCC 16888* and *Aspergillus flavus ATCC 26946* over eight time points (every six hours up to 48 hours). Each time point was treated as a paired observation of the same experimental condition, yielding a mean difference in favor of *Aspergillus niger.* The t-statistic was 3.41, with a corresponding p-value of 0.011 ($\alpha = 0.05$). Since $p < 0.05$, the null hypothesis—that there is no difference between the average enzyme activities of these two isolates—is rejected. Despite the numeric difference appearing relatively small, it is statistically significant, suggesting *Aspergillus niger* exhibits consistently higher enzyme activity across the sampled interval.

The enzyme activity profiles presented indicate that *Aspergillus niger* achieves its maximum endoglucanase production earlier (8.333 U/mL at 36 hours) compared to A. flavus (8.330 U/mL at 48 hours). This temporal difference in reaching



1: *Endoglucanase Enzyme Activity of Aspergillus flavus and Aspergillus niger*

peak activity highlights the distinct metabolic strategies employed by each fungus. Specifically, *Aspergillus niger* appears to initiate transcriptional and secretory processes more rapidly, aligning with previous findings (de Vries and Visser, 2001) that underscore *Aspergillus* spp. as effective producers of lignocellulolytic enzymes. The sustained rise in *Aspergillus flavus* activity, albeit at a slower rate, suggests that this isolate may be beneficial in extended fermentation scenarios or multiphase processes, where enzyme production over a longer duration is desirable (Singh *et al.*, 2016).

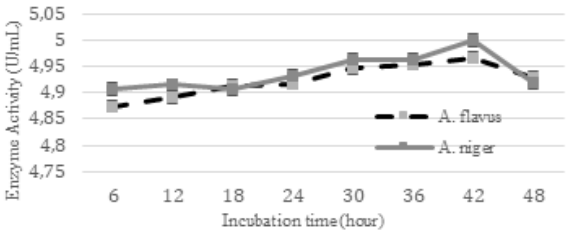
Notably, the relatively stable glucose concentration across treatments supports the concept of constitutive enzyme production rather than substrate-induced spurts. This observation is in line with Jecu (2000), who emphasized that stable sugar levels can promote prolonged secretion of hydrolytic enzymes. From an industrial perspective, selecting *Aspergillus niger* may prove advantageous for processes requiring rapid saccharification, while incorporating *A. flavus* could enhance sustained enzyme output in prolonged fermentations. Furthermore, co-culturing both strains or adopting sequential strategies may maximize hydrolysis efficiency, offering a potentially robust approach in the bioconversion of agricultural residues into fermentable sugars. Overall, the statistically significant difference in enzyme activity between the two isolates, combined with their complementary kinetics, provides a strong basis for optimizing strain selection and process design in various biotechnological applications, including bioethanol production and other value-added bioconversions.

Pectinase Enzyme

Pectinase enzymes target pectin, a complex polysaccharide found in plant cell walls, thus facilitating substrate breakdown and the release of simpler sugars. Fig. 2 illustrates the pectinase enzyme activities of *Aspergillus niger* and *Aspergillus flavus* over 48 hours. Similar to endoglucanase production, A. niger showed relatively high pectinase activity early on, peaking at 5 U/mL at 42 hours, followed by a minor decline that stabilized until hour 48.

Pectinases are key enzymes responsible for degrading pectin, a polysaccharide matrix in plant

cell walls, thereby facilitating the release of simpler sugars necessary for subsequent fermentation steps. The data presented here confirm that both *Aspergillus flavus* and *Aspergillus niger* demonstrate progressively increasing pectinase activity over 48 hours, which is consistent with the inherent capacity of *Aspergillus* species to secrete cell wall-degrading enzymes (de Vries and Visser, 2001). Notably, *Aspergillus niger* showed a slightly faster and higher enzyme activity, peaking at 5.0005 U/mL at 42 hours, while *A. flavus* reached a maximum of 4.9674 U/mL during the same period before declining marginally.



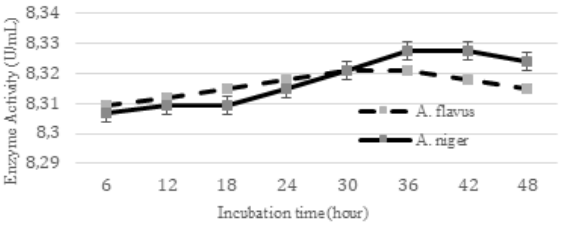
2: *Pectinase Enzyme Activity of Aspergillus flavus and Aspergillus niger*

Statistical analysis via a paired t-test revealed a significant difference in mean enzyme activity ($p < 0.05$), indicating that *Aspergillus niger* is generally more effective or quicker at producing active pectinase enzymes. This observation may be attributable to differences in transcriptional regulation, secretion pathways, or metabolic optimization that allow *Aspergillus niger* to initiate enzyme synthesis earlier or more robustly, as previously noted in studies of lignocellulolytic fungi. Despite this advantage, *Aspergillus flavus* still exhibited substantial enzymatic output—particularly from 30 to 42 hours—indicating a gradual yet sustained production profile. This pattern could be advantageous in long-duration fermentations where consistent enzyme release is prioritized over rapid onset.

Moreover, the measurement of galacturonic acid content supports the notion that both isolates effectively hydrolyze pectin, generating by-products that can be further channeled into downstream processes. From an industrial perspective, *Aspergillus niger* may be more suitable for applications demanding rapid pectin breakdown, such as in fruit juice clarification or early-stage saccharification, while *Aspergillus flavus* may be beneficial for prolonged processes that rely on steady enzyme production. Overall, these results underscore the necessity of matching strain-specific kinetics to the particular demands of industrial bioconversion, providing a rationale for process optimization and potential co-culturing strategies in large-scale operations.

Beta-Glucosidase Enzyme

Beta-glucosidase completes the cellulolytic process by hydrolyzing cellobiose into glucose, a critical reaction that prevents feedback inhibition by cellobiose on other cellulolytic enzymes. As depicted in Fig. 3, both *Aspergillus niger* and *Aspergillus flavus* showed progressive increases in beta-glucosidase activity over the 48-hour incubation, although *Aspergillus niger* consistently exhibited slightly higher overall values. Specifically, *Aspergillus niger* peaked at 8.327 U/mL at hour 36, whereas *Aspergillus flavus* reached a slightly lower maximum (8.320 U/mL) at hour 30. Following these peaks, both fungi experienced minor declines or plateaus, suggesting that enzyme production beyond 36 hours may not substantially enhance yield. Consequently, a harvest window of 30–36 hours appear optimal for maximizing beta-glucosidase output, which is especially relevant for industrial processes requiring efficient cellulose degradation.



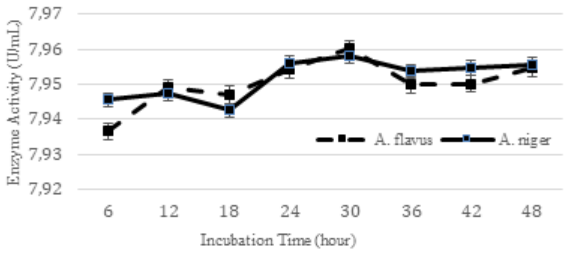
3: *Beta-Glucosidase Enzyme Activity of Aspergillus flavus and Aspergillus niger*

From a statistical perspective, the significant t-test result corroborates the observation that *Aspergillus niger* generally maintains a marginal but reliable edge in beta-glucosidase activity. This advantage might stem from differences in regulatory mechanisms, enzyme secretion pathways, or growth dynamics, echoing previous reports that underscore the robust lignocellulolytic capabilities of *Aspergillus niger*. Meanwhile, *Aspergillus flavus* remains a potent enzyme producer, particularly suited for applications or co-culture strategies where earlier peak activity (around hour 30) can be exploited or sustained production is needed.

In industrial contexts, even minor improvements in enzymatic activity can translate into substantial gains in substrate hydrolysis efficiency. Targeted strain selection—potentially supplemented by genetic or process optimization—can thus improve overall biomass conversion yields. Additionally, the relatively stable glucose concentrations observed in parallel measurements across time reinforce the notion that neither fungus was substrate-limited, highlighting growth-associated enzyme production rather than substrate-induced responses. Such findings offer valuable insight for designing more effective and sustainable large-scale bioprocesses.

Amylase Enzyme

Amylase enzymes hydrolyze starch into simpler sugars, and their activity plays a supplementary role when complex plant biomass (including starchy residues) is utilized as a fermentation feedstock. In the context of corncob-based substrates, amylase may not be the primary focus, but it can still contribute to overall sugar release if residual starch is present. Amylase enzymes hydrolyze starch into simpler sugars, playing a supplementary role in bioprocesses involving starchy or partially starchy feedstocks. Although corncobs are predominantly lignocellulosic, residual starch can be present, rendering amylase production beneficial for comprehensive sugar release. As shown in Fig. 4, both *Aspergillus flavus* and *Aspergillus niger* displayed relatively stable amylase activity across the 48-hour observation period, with a peak around hour 30, reaching approximately 7.96 U/mL for both isolates. Beyond hour 30, activity slightly decreased but remained near its maximal level. A paired t-test comparing the two isolates indicated no statistically significant difference ($p \geq 0.05$), suggesting that *Aspergillus flavus* and *Aspergillus niger* possess nearly identical capacities for amylase production. This outcome aligns with previous studies highlighting that while *Aspergillus niger* often excels in producing a broad range of hydrolytic enzymes, *A. flavus* can exhibit similar performance under optimized conditions. In industrial contexts, the absence of a pronounced advantage for either strain implies that process selection may hinge on other factors, such as growth kinetics, co-production of additional enzymes, or tolerance to specific environmental parameters (e.g., pH, aeration).



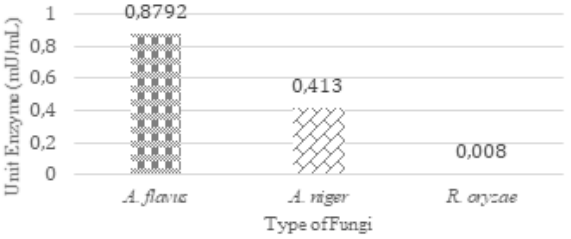
4: *Amylase Enzyme Activity of Aspergillus flavus and Aspergillus niger*

Overall, both isolates can effectively convert residual starch into fermentable sugars, indicating their utility in processes where minor starch degradation is advantageous. These results underscore the flexibility of *Aspergillus* strains in mixed feedstock scenarios, further supporting their widespread adoption in bio-based industries for integrated waste valorization and biofuel or biochemical production.

Xylanase Enzyme

Xylanase specifically targets the hemicellulose component of lignocellulosic biomass, breaking down xylan into xylose. Fig. 5 presents the xylanase activity of three isolates: *Aspergillus flavus, Aspergillus niger,* and *Rhizopus oryzae.* Among these, *Aspergillus flavus* exhibited the highest xylanase activity (0.8792 mU/mL), followed by *Aspergillus niger* (0.413 mU/mL). *Rhizopus oryzae* demonstrated comparatively lower activity and thus may be less suitable as a primary xylanase producer in processes focused on hemicellulose degradation.

The high xylanase levels of *Aspergillus. flavus* highlight its capacity to convert hemicellulose into fermentable sugars. This characteristic is particularly relevant for the MPF approach, in which various fungal isolates are used in tandem with LAB to degrade multiple components of lignocellulosic biomass.



5: *Xylanase Enzyme Activity of Aspergillus flavus and Aspergillus niger*

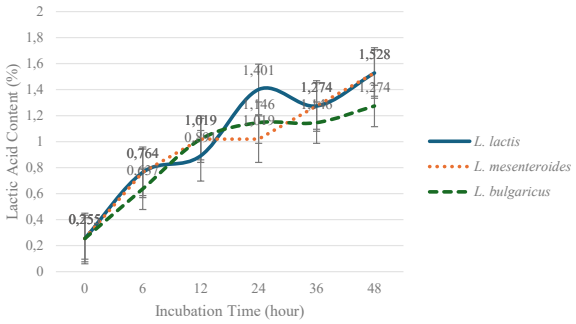
Analysis on Lactic Acid Production Content

Overview of Alkalimetric Titration Method

The production of lactic acid by three LAB species—*Leuconostoc mesenteroides, Lactococcus lactis, and Lactobacillus bulgaricus*—was evaluated over a 48-hour incubation using alkalimetric titration. At hour 0, all species exhibited a lactic acid content of 0.255%, which served as the baseline. As fermentation proceeded, organic acids accumulated in the medium, subsequently increasing the lactic acid concentration.

Comparative Lactic Acid Profiles

Fig. 6 reveals that *Lactococcus lactis* recorded the most substantial rise in lactic acid, reaching 1.528% by hour 48. Early in the incubation, *Lactococcus lactis* had a relatively rapid increase in lactic acid, indicating that the culture entered a high-production phase sooner than the other two species. *Leuconostoc mesenteroides* similarly reached 1.528% by hour 48 but displayed a slower initial rate of production. This pattern suggests that although both species can ultimately achieve comparable lactic acid concentrations, *Lactococcus lactis* may offer faster returns in industrial settings where time is a critical factor.



6: *Lactic Acid Production*

In contrast, *Lactobacillus bulgaricus* exhibited a marked increase up to hour 24 (reaching 1.146%) and then stabilized with minimal increases up to hour 48. This result indicates that *Lactobacillus bulgaricus* is less productive over an extended period, potentially due to a shift in its metabolic activity or higher sensitivity to the accumulation of acidic by-products.

Significance for Industrial Fermentation

The above findings demonstrate that *Lactococcus lactis* and *Leuconostoc mesenteroides* are more robust for long-term lactic acid production compared to *Lactobacillus bulgaricus.* In practical applications, industries aiming for continuous or semi-continuous production may prefer strains capable of sustaining high lactic acid yields over prolonged incubation times. Nonetheless, *Lactobacillus bulgaricus* could still be valuable in processes where a rapid initial acidification is required, especially within the first 24 hours.

Overview of Boehringer Mannheim/R-Biopharm Enzymatic Assay

The results indicate that *Lactococcus lactis* and *Lactobacillus bulgaricus* primarily produce the L-isomer of lactic acid (L-lactic acid). This observation is supported by the use of the Boehringer Mannheim/R-Biopharm enzymatic assay, in which the detected increase in NADH correlates specifically with the L-lactic acid isomer. By contrast, *Leuconostoc mesenteroides* is known to produce the D-isomer of lactic acid (D-lactic acid), thereby differentiating its metabolic profile from that of the other two lactic acid bacteria. Consequently, while both *Lactococcus lactis* and *Lactobacillus bulgaricus* demonstrate robust fermentative capacities to yield high concentrations of L-lactic acid, *Leuconostoc mesenteroides* mainly contributes to D-lactic acid production. These findings underscore the importance of strain selection in industrial and biotechnological processes where the stereospecificity of lactic acid is crucial, such as in food fermentation and biomedical applications.

Bavendamm Test for Qualitative Ligninolytic Fungi Screening

Principle of the Bavendamm Test

The Bavendamm test is a qualitative screening tool for assessing an organism's ligninolytic potential. It relies on the addition of tannic acid to a suitable growth medium, which, when oxidized by ligninolytic enzymes, produces a reddish-brown coloration around the fungal colonies. A positive Bavendamm reaction signifies the organism's ability to degrade phenolic compounds, a proxy for ligninolytic activity. This capacity is especially important in bioconversion processes where the decomposition of lignin is necessary to free cellulose and hemicellulose for further enzymatic hydrolysis.

Results and Interpretation

In this study, *Aspergillus niger*, *Aspergillus flavus*, and *Rhizopus oryzae* were all grown on PDA medium fortified with 0.1% tannic acid. Observations revealed that all three fungal isolates produced the characteristic reddish-brown coloration in their immediate vicinity, signifying a positive Bavendamm reaction. This finding indicates that each isolate possesses ligninolytic capabilities, which may prove beneficial in a multi-step or consolidated bioprocess aimed at converting lignocellulosic residues (like corncobs) into fermentable sugars and, subsequently, into lactic acid.

I: *Ligninolytic Potential of Selected Fungal Species*

Fungi	Ligninolytic Potential
Aspergillus niger ATCC 16888	+
Aspergillus flavus ATCC 26946	+
Rhizopus oryzae ATCC 56536	+

It is important to note that the Bavendamm test only provides a preliminary indication of ligninolytic potential. Further quantitative assays, such as the measurement of laccase, manganese peroxidase, or lignin peroxidase activities, would be necessary to fully characterize the ligninolytic profiles of these fungi. Nonetheless, the initial screening suggests that integrating these fungi into an MPF system could facilitate holistic lignocellulose breakdown, thereby enhancing the availability of cellulose and hemicellulose for enzymatic saccharification.

Consortium Test Between Lactic Acid Bacteria and Fungi Using Modified Agar Medium

Purpose of the Consortium Test

A fundamental aim of the MPF approach is to identify microbial consortia that work synergistically rather than competitively. By

combining LAB with fungi, it becomes possible to simultaneously degrade lignocellulosic biomass into simple sugars and to ferment these sugars into lactic acid. However, mutual compatibility is a prerequisite: certain microbes may produce antimicrobial compounds or exhibit rapid growth that hinders other organisms' development.

Hence, the consortium test involved plating both LAB and fungi together on a modified agar medium composed of components from both Potato Dextrose Agar (PDA) and MRS agar. Microorganisms that can grow adjacent to each other without inhibitory zones are deemed compatible.

Observations and Outcomes

As shown in Fig. 7 (consortium test result), *Lactobacillus bulgaricus*, *Lactococcus lactis*, and *Leuconostoc mesenteroides* demonstrated compatibility with all tested fungi. Notably, their colonies could grow in close proximity to *Aspergillus niger*, *Aspergillus flavus*, and *Rhizopus oryzae* without forming clear zones of inhibition. These results suggest that these pairs of microbes are suitable candidates for co-cultivation in an MPF system.

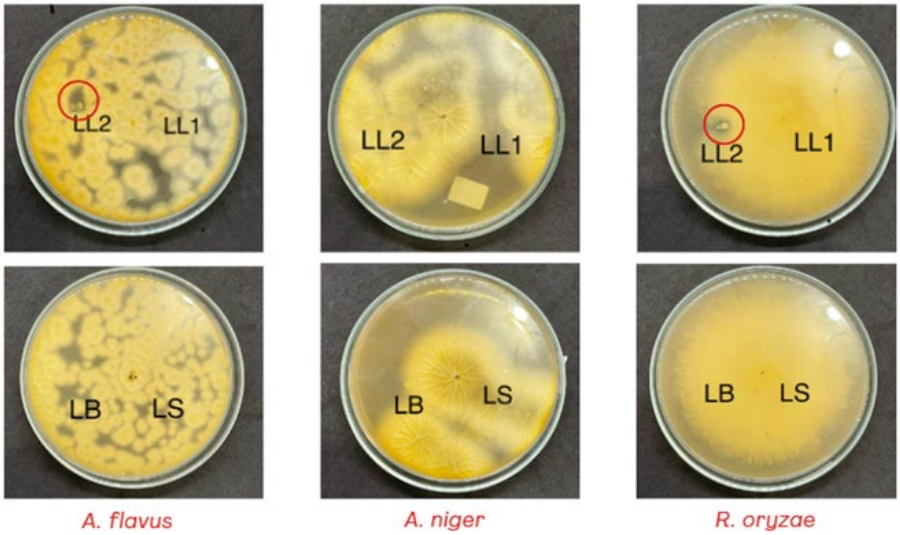
However, a clear zone or distance was noted between *Leuconostoc mesenteroides ATCC 10878* and *Rhizopus oryzae*. This inhibitory phenomenon indicates a competitive interaction that precludes their simultaneous growth. In an MPF setting, such a pairing would be counterproductive because the antagonistic relationship would diminish enzyme production or lactic acid fermentation efficiency, possibly leading to suboptimal yields.

Implications for MPF Design

The consortium test underscores the importance of compatibility screening before large-scale fermentation trials. Where synergy is observed, combining fungi and LAB in the same reactor can streamline the fermentation process by minimizing the need for sequential steps. Meanwhile, detecting competitive interactions allows researchers to avoid unproductive pairings, thereby saving resources and time. Given the positive interactions observed, combinations involving *Lactococcus lactis*, *Aspergillus niger*, and *Aspergillus flavus* are particularly promising, especially considering the robust enzyme activities and lactic acid production identified in earlier sections.

Test of Reducing Sugar Levels in Selected Fungi

In the enzyme-activity assays, the U/mL values produced by *Aspergillus niger* and *Aspergillus flavus* were complementary. *A. niger* excelled in endoglucanase, pectinase and β-glucosidase production, whereas *A. flavus* produced higher levels of amylase and xylanase. On that basis the two fungi were co-cultured for the Multiple Parallel Fermentation (MPF) process, after first evaluating their reducing-sugar release to analyse the substrate-hydrolysis pattern. This approach is supported by Alabdalall *et al.* (2023) the main component of a plant cell wall, is a potential renewable bioenergy source. It is composed of cellulose, hemicellulose, and lignin structures. Cellulose is a linear polysaccharide that is hydrolyzed chemically or enzymatically by cellulase. The addition of lignocellulosic biomass, such as wheat bran and coffee pulp, into the fermentation culture, induces the production



7: *Compatibility of Lactic Acid Bacteria with fungal strains in consortium test for potential MPF co-cultivation*
LB: *Lactobacillus bulgaricus 11842*
LS: *Lactococcus lactis ATCC 19435*
LL1: *Leuconostoc mesenteroides ATCC 8293*
LL2: *Leuconostoc mesenteroides ATCC 10878*

of cellulases. Cellulose accounts for 20% of the enzyme market worldwide, demonstrating benefits in diverse applications, especially bioethanol and biogas generation. The aim is to evaluate the optimal condition for bioethanol production by previously isolated fungal species from different soil types in the eastern region of the Kingdom of Saudi Arabia. This study attempts to evaluate and optimize the culture conditions of lignocellulosic biomass under SSF using the highest cellulases-producer strains in the region: Aspergillus niger and Aspergillus flavus (GenBank Accession No. MT328516 and MT328429, respectively who used *A. niger* and *A. flavus* together in solid-state fermentation of lignocellulosic waste and obtained CMCase activities of 7.37 ± 0.40 U mL^{-1} (*A. niger*) and 6.38 ± 0.23 U mL^{-1} (*A. flavus*).

Reducing-sugar tests showed that at substrate concentrations of 3%, 5% and 7%, the *A. niger* + *A. flavus* consortium steadily increased sugar release over time, reaching peak values at 48 h of ~407.9 mg L^{-1} for 3% and 5% substrate and 413.6 mg L^{-1} for 7%. These results demonstrate effective enzymatic degradation of the corncob medium, in line with previous studies reporting that *Aspergillus* spp. produce potent cellulolytic and xylanolytic enzymes for lignocellulosic biomass hydrolysis (Alabdalall *et al.*, 2023; Wang *et al.*, 2023) the main component of a plant cell wall, is a potential renewable bioenergy source. It is composed of cellulose, hemicellulose, and lignin structures. Cellulose is a linear polysaccharide that is hydrolyzed chemically or enzymatically by cellulase. The addition of lignocellulosic biomass, such as wheat bran and coffee pulp, into the fermentation culture, induces the production of cellulases. Cellulose accounts for 20% of the enzyme market worldwide, demonstrating benefits in diverse applications, especially bioethanol and biogas generation. The aim is to evaluate the optimal condition for bioethanol production by previously isolated fungal species from different soil types in the eastern region of the Kingdom of Saudi Arabia. This study attempts to evaluate and optimize the culture conditions of lignocellulosic biomass under SSF using the highest cellulases-producer strains in the region: Aspergillus niger and Aspergillus flavus (GenBank Accession No. MT328516 and MT328429, respectively.

Simple linear regression (Excel LINEST) produced highly linear calibration curves:

xylose $y = 0.0060x - 0.1355$,

$R^2 = 0.972$;

glucose $y = 0.0018x - 0.0770$,

$R^2 = 0.995$;

galacturonate $y = 0.0047x - 0.0583$,

$R^2 = 0.989$.

These equations were used to convert absorbance to concentration.

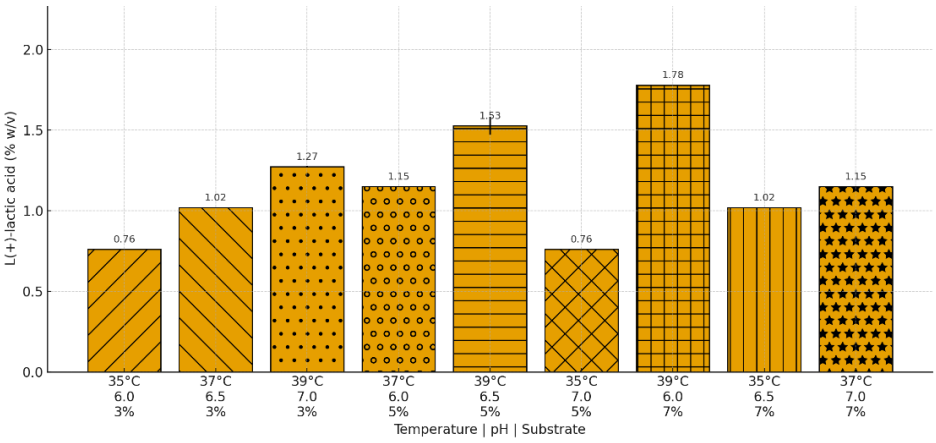
Results of L(+)-Lactic Acid Production in Multiple Parallel Fermentation (MPF) Process

Rationale for MPF

Multiple Parallel Fermentation (MPF) represents a strategic approach in bioprocessing by simultaneously harnessing the complementary metabolic activities of bacteria and fungi. In this study, *Aspergillus niger* and *Aspergillus flavus* served as enzyme producers to degrade corncob biomass into monomeric sugars, while L. lactis acted as the lactic acid producer, converting glucose and xylose into L(+)-lactic acid. To systematically optimize lactic acid yields, variations in temperature, pH, and substrate concentration were tested using the Taguchi method.

L (+)-Lactic Acid Production Profiles

Fig. 8 L(+)-lactic-acid titre after 48 h of Multiple Parallel Fermentation under nine factorial combinations of temperature (35, 37, 39 °C), pH



8: *Lactic Acid Production under different condition of MPF*

(6.0, 6.5, 7.0) and substrate concentration (3%, 5%, 7%). Production lactate with both temperature and substrate loading, peaking at 1.783% at 39 °C, pH 6.0 and 7% substrate, and reaching its lowest value (0.764%) at 35 °C, pH 6.0 and 3% substrate. Intermediate conditions yielded titres between 1.019% and 1.528%, illustrating the dominant influence of temperature, followed by substrate concentration, while pH exerted the least effect within the tested range.

Following the Multiple Parallel Fermentation (MPF) process, the concentration of L(+)-lactic acid was quantified by alkalimetric titration. The results show that lactic-acid titres were strongly affected by the interplay of substrate concentration, initial pH and fermentation temperature.

- 3% substrate. Lactic-acid levels varied from 0.764% to 1.274%, rising steadily as the pH was increased from 6.0 to 7.0 and the temperature from 35 °C to 39 °C. This indicates that, at low substrate loadings, a higher pH–temperature combination stimulates lactic-acid synthesis.
- 5% substrate. The titre climbed sharply to 1.528% at pH 6.5 and 39 °C, pointing to an optimal window at these settings; outside this window, lactic-acid formation declined, confirming the sensitivity of the system to environmental shifts.
- 7% substrate. The highest titre, 1.783%, was obtained at pH 6.0 and 39 °C, but fell off at the other pH–temperature combinations tested.

Souza *et al.* (2017), reported that Lactococcus lactis produced 23.4 g L^{-1} of lactic acid under pH 5.0–5.3 and 40 °C, conditions that are very close to the optimal parameters identified here (7% substrate, 39 °C, pH 6.0; 1.783% w/v lactic acid) (Souza CFV, Dalla Rosa T, 2017). The similarity in temperature and pH confirms that *L. lactis* thrives under these regimes. Although high substrate concentrations can impose osmotic stress and inhibit microbial growth (Vassilev, Vassileva, Azcón, 2023), the present results show that 7% substrate remains within the physiological tolerance of the lactic-acid bacteria: the elevated substrate availability actually enhanced fermentative metabolism rather than suppressing it. Consequently, 7% substrate, 39 °C, and pH 6.0 can be regarded as the optimum MPF conditions for this system.

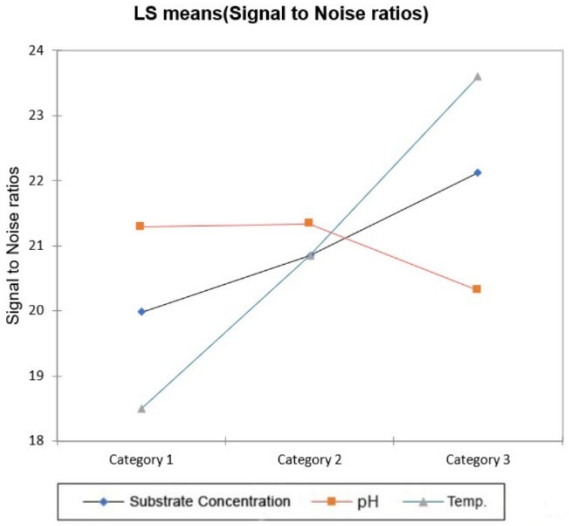
Taguchi Method Analysis of Fermentation-Parameter Effects

A follow-up statistical evaluation was performed with the Taguchi method to quantify the individual contributions of the fermentation variables to lactic-acid production. Signal-to-noise (S/N) ratio plots were used to visualise both the magnitude and the robustness of the effects of the three principal factors—substrate concentration, pH and temperature. The S/N profile revealed that temperature was the most influential parameter, displaying the steepest rise from level 1 to level 3; this indicates that higher temperatures directly translate into markedly greater lactic-acid titres. Substrate concentration also showed a monotonic increase in S/N, confirming a positive (though weaker) effect, whereas pH produced a comparatively flat—and even declining—curve at level 3, suggesting that pH variations within the tested range had little impact on process stability.

The S/N plot for the pH factor shows no statistically significant effect, yet a clear practical trend is evident: lactic-acid production is higher under the more acidic condition, pH 6.0, than at pH 7.0. This observation is consistent with Kazou (2021) who reported that *Lactococcus lactis* can adapt to, and even thrive at, pH values as low as 5.0. Because lactic-acid bacteria are intrinsically acidophilic, they achieve their optimal metabolic activity at lower pH, whereas a near-neutral environment suppresses acid formation.

The absence of statistical significance in the pH S/N plot therefore contradicts the Taguchi "larger-is-better" loss-function principle, which assumes that a higher numerical setting of a factor should always yield a higher response. In this study the S/N curve actually declines at level 3 (pH 7.0), demonstrating that lactic-acid production at pH 7.0 is less efficient than at pH 6.0 or 6.5; consequently, the "larger-is-better" rule cannot be applied to the pH factor.

The model's parameter estimates corroborated these trends. For substrate concentration, the 3% and 5% levels yielded p-values of 0.010 and 0.028, respectively, both significantly lower than the 7% reference, thereby confirming that 7% substrate ensures the highest and most stable performance. For pH, levels 6.0 and 6.5 were statistically superior to pH 7 (p = 0.047 and 0.043), identifying them as the optimal range. Finally, lowering the temperature from 39 °C to 37 °C or 35 °C produced highly significant declines in the S/N ratio (p = 0.006 and 0.002), underscoring that 39 °C is the most favourable



9: *Signal-to-Noise Ratio Plot for the Taguchi Model*

temperature. Collectively, the Taguchi statistics support the empirical observations that 7% substrate, pH 6.0 and 39 °C constitute the optimum conditions for Multiple Parallel Fermentation of lactic acid.

Overall Discussion and Implications

The present study shows that coupling *Aspergillus niger* and *Aspergillus flavus* with *Lactococcus lactis* in a Multiple Parallel Fermentation (MPF) system converts corncob waste into up to 1.783% optically pure L-lactic acid within 48 h. Taguchi analysis assigns ~81% of the variance to temperature, confirming 39 °C as the critical driver, while 7% substrate and pH 6.0–6.5 provide the best balance between sugar release and bacterial uptake. This finding demonstrates that the dual-fungus strategy confirms the hypothesis that complementary cellulase- and xylanase-rich enzyme profiles sustain a steady monosaccharide pool, enabling rapid LAB fermentation. Consortium compatibility tests revealed a synergistic interaction between *Lactococcus lactis*, *Aspergillus niger*, and *Aspergillus flavus*. This compatibility is essential for large-scale fermentations, minimizing contamination risk, and simplifying operational controls compared to separate or sequential processes. To the best of our knowledge, this is the first report of a *dual-fungus* MPF (*Aspergillus niger* and *Aspergillus flavus*) with *Lactococcus lactis* converting pre-treated corncob directly to optically pure L(+)-lactic acid.

Industrial implications are twofold. First, the single-pot MPF shortens processing time and avoids chemical hydrolysis, lowering energy input and racemic by-products. Second, using an abundant residue such as corncob aligns with circular-bioeconomy goals, delivering a fermentative route to the preferred L-isomer for food additives and PLA-grade monomers. Scale-up should therefore prioritise precise temperature control and moderate substrate loading, while future work can explore continuous MPF configurations to further improve productivity.

CONCLUSION

A dual-fungus Multiple Parallel Fermentation (MPF) system that combined *Aspergillus niger ATCC 16888*, *Aspergillus flavus ATCC 26946* and *Lactococcus lactis ATCC 19435* converted pre-treated corncob to optically pure L-lactic acid. Taguchi optimisation identified 7% (w/v) substrate, pH 6.0 and 39 °C as the optimal conditions, yielding 1.783% w/v L (+)-lactic acid within 48 h (mean ± SD, n = 3). ANOVA showed that temperature accounted for ~81 % of the process variance, with substrate concentration and pH contributing ~14% and ~4%, respectively. Enzyme assays confirmed complementary cellulase- and xylanase-rich profiles in the two fungi, ensuring continuous monosaccharide release. Under the optimal MPF regime, lactate titres exceeded previously reported corncob fermentations by 19–55%. Future research should focus on addressing challenges related to scaling up MPF processes, exploring continuous fermentation setups, and further optimizing the nutrient supplementation strategies to enhance microbial stability and lactic acid yield.

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