

Enhanced *Rhizomucor miehei* Lipase Production through Morphological and Fermentation Optimization in *Aspergillus oryzae*

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Abstract

Rhizomucor miehei lipase (RML) is widely applied in food processing, lipid modification, and biocatalysis; however, the scale-up of its heterologous production in *Aspergillus oryzae* (*A. oryzae*) is constrained by excessive mycelial aggregation, oxygen limitation, and unfavorable broth rheology. An integrated morphology and fermentation engineering strategy was developed to improve RML production in recombinant *A. oryzae*. First, at the shake-flask level, talc addition facilitated the formation of uniform pellets, with an optimal mean pellet diameter of approximately 0.7 mm and a maximum RML activity of 289.0 U/mL. Subsequently, a staged agitation program was established in a 5-L bioreactor fermentation to maintain the target pellet size, increasing RML activity to 486.5 U/mL, which was 70.7% higher than that of the control (285.0 U/mL). Comparative intracellular metabolite profiling showed that the optimized process was accompanied by increased levels of serine and threonine, which are the two most abundant amino acid residues in RML, together with glutamine. These metabolic changes suggested an improved supply of amino acid building blocks for protein biosynthesis and provided a possible metabolic explanation for the enhanced RML accumulation. Continuous feeding was introduced to sustain nutrient availability during the mid- to late-fermentation stages, thereby accelerating RML accumulation and increasing the final activity to 570.0 U/mL. Finally, late-stage pH control at 8.0 improved pellet stability and further enhanced RML production, yielding 742.0 U/mL, equivalent to 2.6-fold the control. Overall, this integrated strategy provides a practical framework for the bioreactor production of recombinant lipases by filamentous fungi.

1. Introduction

Lipases (EC 3.1.1.3) are important industrial biocatalysts that catalyze triglyceride hydrolysis, esterification, and transesterification and are extensively used in food, pharmaceutical, detergent, fine-chemical, and biodiesel applications (Chandra et al., 2020; Contesini et al., 2020; Eskandari et al., 2024; Kumar et al., 2023; Mahfoudhi et al., 2022; Yao et al., 2021). With the continued development of green manufacturing and enzymatic processes, efficient, stable, and scalable lipase production platforms have become a major focus of industrial biotechnology. *Rhizomucor miehei* lipase (RML) combines favorable catalytic performance with operational robustness, giving it substantial commercial potential as a fungal lipase (Rodrigues and Fernandez-Lafuente, 2010; Tian et al., 2022). As a typical sn-1,3-regioselective lipase, RML exhibits strong regioselectivity toward triglycerides, making it particularly valuable for structured-lipid synthesis, targeted lipid modification, and food-oil processing (Rodrigues and Fernandez-Lafuente, 2010; Tian et al., 2022; Wang et al., 2026).

Aspergillus oryzae (*A. oryzae*) has a food-grade safety record, a strong capacity for extracellular protein secretion, and a well-established foundation in industrial fermentation, making it an attractive host for recombinant enzyme production (Li et al., 2022; Matsumoto et al., 2021; Zhang et al., 2021). However, filamentous fungi display complex morphologies during submerged fermentation, ranging from dispersed hyphae and flocculent aggregates to compact pellets. These morphological states directly affect broth rheology, oxygen transfer, substrate diffusion, and product secretion and are therefore

among the principal determinants of filamentous fungi fermentation performance (Gong et al., 2023; Lu et al., 2024; Meyer et al., 2021; Papagianni, 2004; Wucherpennig et al., 2010). During 5-L bioreactor fermentation of heterologous RML production by *A. oryzae*, excessive mycelial aggregation, adhesion to the agitator shaft and vessel wall, insufficient dissolved oxygen, and loss of effective working volume frequently reduce volumetric enzyme activity and hinder further industrialization (Li et al., 2022; Nienow, 1998; Zhang et al., 2021).

Morphology engineering has consequently emerged as an important strategy for improving filamentous fungi fermentation. Manipulating spore concentration, microparticle addition, agitation intensity, aeration, and medium composition can reshape pellet formation and, in turn, improve product synthesis (Driouch et al., 2010; Gong et al., 2023; Ichikawa et al., 2022; Kowalska et al., 2020; Lu et al., 2024; Meyer et al., 2021; Singh, 2018; Yatmaz et al., 2020). Otherwise, feeding regimes and culture pH can likewise alter fungal morphology and secretory metabolism by modulating carbon and nitrogen metabolism, osmotic pressure, and cell-surface charge (Rahimzadeh et al., 2024; Venkatachalam et al., 2023). Although microparticle-enhanced cultivation, agitation-shear optimization, and fed-batch strategies have each been reported to improve enzyme production in filamentous fungi (Ichikawa et al., 2022; Kowalska et al., 2020; Li et al., 2022; Rahimzadeh et al., 2024; Singh, 2018; Venkatachalam et al., 2023; Yatmaz et al., 2020; Zhang et al., 2021), the coordinated regulation of morphology, nutrient supply, and pH during bioreactor fermentation, together with its relationship to intracellular metabolic responses, remains insufficiently understood.

In this study, an integrated process was established for RML production by recombinant *A. oryzae*. Microparticle addition and agitation speed were first evaluated in shake flasks to identify a pellet morphology conducive to enzyme production. Dynamic agitation was then applied in a 5-L bioreactor fermentation to regulate morphology and oxygen availability during fermentation. Intracellular metabolite profiles from the control process (RY0) and the optimized dynamic agitation process (RY1) were compared to elucidate the possible explanation of the improvement. Continuous feeding was subsequently introduced to stabilize nutrient supply, after which several late-stage pH trajectories were tested to determine how intervention timing and set point affected RML production and pellet stability. Together, these experiments established an integrated framework for improving recombinant lipase production in filamentous fungi bioreactors.

2. Materials and methods

2.1. Strain, media, and spore preparation

The RML-expressing recombinant strain *Aspergillus oryzae* CGMCC 3.16200 (III3-5C-1), deposited at the China General Microbiological Culture Collection Center (CGMCC), was used throughout this study. The strain was inoculated onto potato dextrose agar (PDA; 40 g/L) plates and incubated at 28°C for 48–60 h. After cultivation, spores were dislodged with sterile water and glass beads under agitation. The

suspension was filtered through filter paper and adjusted to 3×10^5 spores/mL for inoculation of the shake-flask seed culture.

The shake-flask seed medium contained (g/L) corn dextrin, 20; corn steep liquor powder, 10; yeast extract, 10; KH_2PO_4 , 1.244; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.249; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 6.0; and antifoam powder, 1.0. The shake-flask fermentation medium contained (g/L) corn dextrin, 50; soy peptone, 30; yeast extract, 4; KH_2PO_4 , 1.12; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.227; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 5.33; and antifoam powder, 1.0, with an initial pH of 6.0. The basal medium for the 5-L bioreactor fermentation contained (g/L) corn dextrin, 5; soy peptone, 3; yeast extract, 0.4; KH_2PO_4 , 1.12; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.227; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 5.33; and antifoam, 1.0, with an initial pH of 6.0. The corn dextrin solution was sterilized separately and added aseptically after the sterilized bioreactor medium had cooled.

2.2. Preliminary investigation of pellet morphology

Seed medium (50 mL) was dispensed into 250-mL shake flasks and sterilized at 121°C for 30 min. Each flask was inoculated with 300 μL of the spore suspension and incubated at 28°C and 150 rpm for 24 h. The resulting seed culture was transferred at 10% (v/v) into the shake-flask fermentation medium and cultivated at 28°C for 120 h. To examine the combined effects of microparticle size, dosage, and agitation speed on pellet formation and RML synthesis, 200- or 400-mesh talc was added to final concentrations of 1, 3, 5, or 10 g/L; a control without talc was included. Each condition was tested at 150 and 200 rpm. At the end of fermentation, RML activity, residual sugar, and pellet morphology were determined. For morphological analysis, 1 mL of fermentation broth was mixed with 1 mL of formaldehyde/ethanol fixative (1:3, v/v), washed with ultrapure water, adjusted to a final volume of 10 mL, spread across a 90-mm Petri dish, and photographed. Mean pellet diameter and pellet concentration were quantified using Image-Pro Plus 6.0.

2.3. Bioreactor cultivation and dynamic agitation strategies

Bioreactor experiments were performed in a 5-L bioreactor fermentation equipped with a Biostar online monitoring system. The initial working volume was 2.5 L, and 250 mL of a 24-h seed culture was used as the inoculum. The temperature was maintained at 28°C, aeration was set to 1 volume of air per volume of liquid per minute (vvm), the initial pH was 6.0, and the total fermentation time was 144 h. The pH and dissolved oxygen (DO) were monitored online, while O_2 and CO_2 in the exhaust gas were monitored by process mass spectrometry. Samples were collected every 24 h to determine RML activity, extracellular protein concentration, dry cell weight (DCW), mean pellet diameter, and pellet concentration offline.

Protein concentration, DCW, mean pellet diameter, and pellet concentration were measured offline. Three agitation strategies were compared to examine how agitation intensity and the timing of speed increases affected pellet morphology, oxygen transfer, and RML synthesis. RY0 represented the control process, RY1 represented the dynamically optimized process, and RY2 represented the intensified-dispersion process. All groups started at 300 rpm and were adjusted stepwise according to Fig. 2a. The RY0 speed was raised successively to 325, 450, 550, and 600 rpm; RY1 was raised to 375, 450, 500, 600, 650, and 700 rpm; and RY2 was raised to 375, 450, 500, 600, 700, 750, and 800 rpm. Agitation was held

constant between adjacent adjustment points. RY0 used relatively gradual speed increments and served as the control fermentation process. RY1 intensified mixing primarily during 24–60 h to improve oxygen transfer during pellet establishment while maintaining moderate shear thereafter. RY2 increased agitation earlier and to a greater extent than RY1 to promote sustained hyphal dispersion and oxygen transfer. DO, pellet size and concentration, extracellular protein accumulation, and RML activity were compared among the three programs to evaluate their shear-oxygen-transfer tradeoffs.

2.4. Continuous feeding and late-stage pH control

The feed medium contained (g/L) corn dextrin, 45; soy peptone, 27; yeast extract, 3.6; and antifoam, 1. In the control feeding protocol, 150 mL of feed medium was added every 12 h. The continuous-feeding process RY3 was developed from RY1 by supplying sterile feed medium continuously at 12 mL/h from 18 to 120 h. Temperature, aeration, dynamic agitation, and all other operating conditions in RY3 were identical to those in RY1.

Three late-stage pH-control strategies were evaluated using RY3 as the common control process. In RY4, the early pH trajectory was kept unaltered; once the pH rose naturally to 7.0, it was maintained at 7.0 with 3 M HCl. In RY5, control was initiated at 96 h, when the process pH was approximately 7.5, and the pH was subsequently maintained at 7.5 with 3 M HCl. In RY6, the pH was allowed to rise naturally to 8.0 and was then maintained at 8.0 with 3 M HCl. The medium, feeding mode, dynamic agitation profile, temperature, and aeration were otherwise identical among RY4, RY5, and RY6.

2.5. Fermentation-process analyses

RML activity was assayed colorimetrically with p-nitrophenyl palmitate (pNPP) as the substrate. A 5-mL aliquot of freshly prepared pNPP working solution was mixed with 45 mL of substrate buffer. Aliquots of 2.4 mL were dispensed into tubes and preincubated at 37°C for 5 min. Appropriately diluted fermentation supernatant (100 µL) was added, and the reaction was conducted at 37°C for 15 min before being terminated with 2 mL of absolute ethanol. The samples were centrifuged at 4,000 rpm for 5 min, and absorbance of the supernatant was measured at 410 nm. One unit was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol per minute under these conditions. Volumetric activity was expressed as U/mL of fermentation broth.

Extracellular protein was quantified at 595 nm using the Coomassie Brilliant Blue assay. DCW was determined by filtering 10 mL of fermentation broth, washing the retained biomass, and drying it to constant weight. Exhaust-gas O₂ and CO₂ data were combined with the aeration rate to calculate the oxygen uptake rate and carbon dioxide evolution rate. The respiratory quotient (RQ), defined as the ratio of carbon dioxide evolution rate to oxygen uptake rate, was evaluated together with DO to characterize oxygen availability and respiratory activity.

2.6. Intracellular metabolite extraction and gas chromatography-mass spectrometry analysis

To compare intracellular metabolism between RY0 and RY1, fungal biomass was sampled at 24, 72, 96, 120, and 144 h. A 1.5-mL broth aliquot was rapidly quenched in precooled 40% (v/v) methanol, washed with precooled methanol, and extracted with 75% ethanol containing an internal standard. Concentrated extracts were used for amino-acid and sugar-phosphate analyses. Amino-acid samples were derivatized with acetonitrile and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide. Derivatives were analyzed by gas chromatography-mass spectrometry (GC-MS), and metabolite contents were normalized to DCW and expressed as $\mu\text{mol/g}$ DCW. Relative amino-acid abundance was compared across all five sampling times, primarily at 24 and 144 h.

3. Results and Discussion

3.1. Identification of a pellet morphology conducive to RML production in shake flasks

To identify shake-flask culture conditions that support both pellet formation and RML biosynthesis, the combined effects of microparticle (talc) addition and shaking speed were systematically evaluated. The interaction between the two variables markedly modulated hyphal aggregation and pellet architecture, giving rise to corresponding differences in RML activity.

For 200-mesh talc, RML activities at 200 rpm were consistently higher than at 150 rpm across all tested concentrations. At 200 rpm, the talc-free control already showed relatively high activity, and 1 g/L 200-mesh talc caused no further enhancement, while concentrations of 3–10 g/L significantly reduced RML activity, likely due to excessive disruption of hyphal aggregation and pellet structure by high loadings of coarse particles. In contrast, 400-mesh talc exhibited a different response pattern. At 150 rpm, supplementation with 1 g/L 400-mesh talc yielded the highest RML activity of 289.0 ± 0.9 U/mL, which was superior to both the talc-free control at 200 rpm and the culture containing 1 g/L 200-mesh talc at 200 rpm. Conversely, at 200 rpm, the addition of 1 g/L 400-mesh talc conferred no further improvement in enzyme production. These results indicate that particle size must be appropriately matched to shaking speed, with finer particles enhancing RML production at low agitation but conferring no benefit at higher speeds (Fig. 1a, b).

To further resolve the relationship between pellet morphology and enzyme production, three high-activity conditions were compared: no talc at 200 rpm, 1 g/L 200-mesh talc at 200 rpm, and 1 g/L 400-mesh talc at 150 rpm. Residual sugar concentrations under the first two conditions were 4.0 ± 0.1 and 4.1 ± 0.1 g/L, respectively. Both cultures contained many relatively small pellets, with concentrations of $2,432.0 \pm 134.1$ and $2,583.0 \pm 77.2$ pellets/mL and mean diameters of 0.3 ± 0.1 and 0.4 ± 0.1 mm, respectively. By contrast, the culture containing 1 g/L 400-mesh talc at 150 rpm retained more residual sugar (5.3 ± 0.2 g/L) but achieved the highest RML activity (289.0 ± 0.9 U/mL). This culture contained fewer pellets ($1,179.0 \pm 92.3$ pellets/mL), with a larger mean diameter (0.7 ± 0.1 mm; Table 1). Thus, under the present conditions, higher RML activity was not simply associated with greater sugar consumption; instead, it

was more plausibly linked to pellet morphology, particularly pellet size, abundance, and internal architecture.

Table 1
Key process parameters for recombinant lipase fermentation under representative high-activity shake-flask conditions.

Condition	Enzyme activity (U/mL)	Residual sugar (g/L)	Pellet concentration (pellets/mL)	Mean pellet diameter (mm)
No talc, 200 rpm	270.0 ± 2.0	4.0 ± 0.1	2432.0 ± 134.1	0.3 ± 0.1
1 g/L 200-mesh talc, 200 rpm	266.0 ± 0.7	4.1 ± 0.1	2583.0 ± 77.2	0.4 ± 0.1
1 g/L 400-mesh talc, 150 rpm	289.0 ± 0.9	5.3 ± 0.2	1179.0 ± 92.3	0.7 ± 0.1

Morphological examination confirmed pronounced differences in pellet size, abundance, and architecture among the microparticle-agitation combinations. Pellets produced without talc at 200 rpm or with 1 g/L 200-mesh talc at 200 rpm were numerous and small, with densely packed internal hyphae and limited peripheral extension. In contrast, those generated with 1 g/L 400-mesh talc at 150 rpm were larger and less numerous, exhibited more extensive peripheral hyphal growth, and had comparatively loose architecture (Fig. 1c, **see the supplementary material**). Therefore, in the *A. oryzae*-RML system, the most favorable morphology was not defined by the smallest pellets alone; rather, it depended on a pellet architecture that balanced oxygen transfer with secretory activity.

Within the investigated range, 1 g/L 400-mesh talc at 150 rpm generated the most favorable pellet morphology and the highest RML activity. The likely role of talc was to modulate the initial hyphal aggregation process, producing pellets with a balanced size and relatively loose architecture that supported oxygen and substrate transfer while maintaining secretory capacity. Although talc was not added during the 5-L bioreactor fermentation experiments, these results defined the target pellet morphology and guided the subsequent coordinated application of dynamic agitation, continuous feeding, and late-stage pH control to reproduce and maintain a high-production state.

3.2. Dynamic agitation regulation alleviated oxygen limitation and enhanced RML production

Having identified a favorable pellet morphology in shake flasks, we next tested whether alternative agitation programs could reproduce that morphology during 5-L bioreactor fermentation. The control process was designated RY0. Given that RY0 was characterized by secondary pellet aggregation, increasing pellet size, and inadequate mid- to late-stage oxygen transfer, two dynamic programs, RY1 and RY2, were designed (Fig. 2a). RY1 was intended to delay oxygen limitation while stabilizing the pellet architecture, thereby matching morphology and oxygen supply to the requirements of RML secretion.

RY2 imposed still greater shear and oxygen transfer to test whether smaller, more dispersed pellets would further favor RML synthesis. The agitation profiles produced distinct DO trajectories. In RY0, DO rapidly approached zero, indicating early oxygen limitation. The decrease was substantially delayed in RY1 and was delayed still further in RY2, which maintained high DO until the mid- to late-stage of fermentation (Fig. 2b).

RML activities were similar among the three processes during early fermentation but diverged after 48 h. At 144 h, RY1 achieved the highest activity of 486.5 U/mL, compared with approximately 391.0 U/mL in RY2 and 285.0 U/mL in RY0. Although RY2 maintained the highest DO, its RML activity remained lower than that of RY1, indicating that greater oxygen availability alone did not maximize enzyme production. Extracellular protein accumulation showed a similar trend. RY1 experienced the largest increase during the middle and late stages of fermentation, reaching its highest concentration at 144 h, followed by RY2 and RY0. (Fig. 2c, d)

These differences were closely associated with pellet morphology. RY0 formed large pellets during 24–72 h and showed a subsequent tendency toward further aggregation. In contrast, RY2 produced numerous small, highly dispersed pellets. RY1 maintained intermediate pellet diameters and concentrations during mid- to late-stage fermentation, resulting in a more stable particle distribution (See the supplementary material). Moreover, the pellet size maintained in RY1 was close to the favorable morphology identified in shake-flask cultures. Overall, RY1 provided a more favorable combination of oxygen availability, pellet stability, and secretory activity than either RY0 or RY2. These results suggest that, under the tested conditions, moderate dynamic agitation was more effective than maximal agitation intensity or DO in maintaining the target morphology and promoting RML production. RY1 was therefore selected as the basis for subsequent process optimization.

3.3. Metabolic Profiling Identified Amino-Acid Changes Associated with Enhanced RML Production

To elucidate the metabolic underpinnings of improved RML biosynthesis achieved via dynamic agitation regulation, intracellular amino acid pool dynamics were profiled and compared between RY1 and RY0 (Fig. 3). While most amino acids exhibited largely congruent temporal patterns across the two conditions, serine, threonine, and glutamine displayed pronounced stage-dependent divergence. In RY1, serine and threonine accumulated progressively from mid to late fermentation, attaining 25.3% and 36.8% higher levels, respectively, relative to RY0 at 144 h. The early-phase reduction in multiple amino acids, occurring before the onset of rapid RML accumulation, primarily reflected metabolic turnover supporting biomass formation and endogenous protein synthesis. The late-stage enrichment of serine and threonine might reflect improved precursor availability under the optimized shear and mixing conditions. Notably, serine and threonine collectively constitute 19.5% of the 363-residue RML precursor polypeptide, highlighting their substantial representation in the target protein (See the supplementary material). The temporal alignment of their accumulation with peak RML synthesis is consistent with a more permissive precursor supply environment in RY1.

Glutamine exhibited stage-specific remodeling of nitrogen metabolism, with levels remaining low at 24 h, increasing markedly after 72 h, and reaching 16.6% higher in RY1 than in RY0 at 144 h — a trajectory that coincided with robust RML biosynthesis. Glutamate, by contrast, showed minimal overall fluctuation. Rather than triggering non-specific accumulation of nitrogen-containing amino acids, dynamic agitation preserved a homeostatically balanced glutamine-glutamate node, which sustains amino group donation and intracellular nitrogen buffering capacity. Collectively, RY1 was associated with stage-specific precursor allocation rather than a global elevation of intracellular amino acid pools. When integrated with the process performance data in Section 3.2, these findings support a working model in which optimized shear-mixing regimes stabilize pellet architecture, enhance oxygen and substrate transfer, and coordinate carbon and nitrogen precursor provision to match the biosynthetic demand of RML.

3.4. Continuous feeding improved process stability and increased RML production

Although RY1 improved pellet morphology and oxygen availability, RML accumulation remained strongly stage dependent, with limited production during the early phase and a marked increase only during mid- to late-stage fermentation. This pattern suggested that nutrient limitation may have restricted early enzyme production and delayed subsequent RML accumulation. Therefore, process development focused on improving nutrient availability through an alternative feeding strategy. The continuous-feeding process RY3 was established on the basis of RY1 and compared with RY1 in terms of RML production, pellet evolution, and other process variables.

Continuous feeding substantially altered biomass formation and enzyme production. At 24–48 h, DCW was higher in RY3 than in RY1, indicating that continuous nutrient delivery accelerated early biomass establishment. The difference subsequently narrowed, and DCW was similar in both processes by 96 h (Fig. 4a). Consistent with the early biomass response, RML activity began to diverge after 48 h and was markedly higher in RY3 at 72 and 96 h. By 120 h, RY3 reached approximately 580.0 U/mL, whereas RY1 reached only approximately 210.0 U/mL, indicating that continuous feeding accelerated the transition to rapid RML production rather than merely increasing the final level (Fig. 4b). Extracellular protein concentrations were consistently higher in RY3 from 72 to 120 h, with the largest differences at 96 and 120 h, further indicating enhanced protein secretion (Fig. 4c). The pH of RY3 also increased more smoothly and fluctuated less during the middle stage than that of RY1, consistent with improved process stability under continuous nutrient supply (Fig. 4d). During late fermentation, however, the pH of RY3 continued to rise and reached approximately 9.1 at 144 h; DCW declined slightly, and RML activity decreased from approximately 580.0 to 570.0 U/mL over the same interval. The temporal concordance of these changes suggested that excessive late-stage alkalization might have been associated with impaired hyphal integrity and reduced sustained enzyme production.

Continuous feeding also altered pellet evolution. Relative to RY1, RY3 generally maintained a greater pellet concentration and smaller mean pellet diameter, with the differences most pronounced during mid- to late-stage fermentation. At 144 h, the RY3 culture retained more pellets with a smaller mean

diameter, indicating reduced late-stage pellet coarsening and a more uniform particle distribution (Fig. 4e, f). This pattern did not imply that smaller or more numerous pellets inherently favored enzyme production; rather, it suggested that maintaining a stable and uniform pellet population might have reduced aggregation and culture heterogeneity, thereby helping preserve conditions conducive to enzyme production.

Continuous feeding therefore further improved 5-L bioreactor fermentation performance when combined with the optimized RY1 agitation program. It accelerated biomass establishment, advanced RML synthesis and extracellular protein secretion, stabilized pellet evolution and the mid-stage pH trajectory. RY3 consequently exhibited faster RML accumulation and greater final activity. The late-stage association between increasing pH and modest declines in DCW and RML activity nevertheless identified excessive alkalization as a potential constraint, providing the rationale for subsequent optimization of late-stage pH control.

3.5. RML production further enhanced through precise late-stage pH control

Because continuous feeding improved mid- to late-stage performance but the pH continued to rise as RML accumulation plateaued, late-stage alkalization was investigated as a potential remaining constraint. As shown in Section 3.4, RML activity increased as the broth pH rose to approximately 8.0, but did not increase further when the pH continued to rise; instead, activity subsequently plateaued and slightly declined. Thus, pH 8.0 was selected as the upper limit of the late-stage control gradient. Rather than fixing the pH throughout fermentation, we allowed it to follow its natural trajectory during the early and productive stages and imposed control only at the late-stage, testing set points of 7.0, 7.5, and 8.0 (RY4, RY5, and RY6, respectively; Fig. 5a). Because the intervention time and the pH set point were varied simultaneously, the effects of these factors could not be completely separated. The three trajectories were therefore compared as integrated late-stage pH-control strategies.

The intervention window was therefore placed in the late production stage, when the pH continued to rise while RML activity approached a plateau, thereby avoiding interference with early growth and secretion. The effects of the pH trajectories emerged primarily during mid- to late- stage fermentation. RML activities were similar among the three processes before 72 h but diverged after 96 h, when RY6 began to outperform RY4 and RY5; this advantage increased further at 120 h. At 144 h, RY6 achieved the highest activity, 742.0 U/mL, compared with approximately 620.0 U/mL in RY4 and 600.0 U/mL in RY5 (Fig. 5b). Allowing the pH to rise naturally and then imposing precise late-stage control at pH 8.0 was therefore associated with the highest RML production under the tested trajectories.

Early biomass levels were similar among the three processes, indicating that the alternative late-stage pH strategies did not materially affect initial growth. During 96–120 h, RY6 maintained a relatively high DCW, suggesting greater biomass retention during the principal enzyme-production stage. At 144 h, DCW was similar in RY5 and RY6 and higher than in RY4, whereas RML activity remained considerably lower in

RY5 than in RY6 (Fig. 5c). Accordingly, the production difference could not be explained by biomass concentration alone and was more plausibly linked to differences in late-stage secretory status and pellet stability. RQ remained close to 1.0 for most of fermentation and differed little among the three processes (Fig. 5d), indicating that the pH-control strategies did not induce a pronounced shift in the overall respiratory metabolic state. The late-stage differences in RML activity were therefore more consistent with process-environment and morphological responses than with a major redirection of basal metabolism.

Pellet morphology further clarified the effect of late-stage pH control. At 96 h, mean pellet diameters were small and similar among the processes. By 120 h, the differences were clear. After the pH had risen naturally, maintaining RY5 at pH 7.0 led to a sharp increase in mean pellet diameter, reaching approximately 1.2 mm, while pellet concentration declined to about 60.0 pellets/mL. These changes suggested pronounced pellet coarsening and an imbalance in pellet structure. In contrast, RY4 and RY6, controlled after the pH rose naturally to 7.5 and 8.0, respectively, retained mean pellet diameters of approximately 0.54–0.56 mm and pellet concentrations of approximately 680.0–690.0 pellets/mL at 120 h (Fig. 5a, e, f). Thus, in this system, the high-production morphology was characterized less by minimizing particle size than by avoiding abrupt coarsening and structural destabilization during the principal production stage. Together, these results indicate that pH control should be aligned with the natural progression of the culture rather than imposed prematurely. Early or poorly timed intervention can disrupt pellet-size distribution, whereas a late-stage adjustment helps maintain a morphology conducive to secretion.

Overall, pH optimization followed a principle of minimizing early interference while timing the late-stage shift deliberately. Holding the culture prematurely at a neutral-to-alkaline pH may disrupt the transition from growth to secretion and compromise subsequent production, whereas a targeted late-stage switch after continuous feeding and morphology optimization appears to preserve a more favorable pellet architecture and secretory environment. RY6 consequently achieved the highest RML activity, underscoring the importance of the late-stage pH trajectory rather than pH elevation alone.

4. Conclusions

An integrated process combining pellet morphology control, continuous feeding, and precise late-stage pH regulation was established to improve RML production by recombinant *A. oryzae*. In shake flasks, 1 g/L 400-mesh talc at 150 rpm generated relatively loose pellets with a mean diameter of approximately 0.7 mm and yielded 289.0 U/mL RML. In a 5-L bioreactor fermentation, the RY1 dynamic agitation program delayed DO depletion and stabilized pellet size and concentration, increasing the 144-h RML activity to 486.5 U/mL, 70.7% above the control process. Continuous feeding in RY3 further accelerated mid- to late-stage RML accumulation, raising the final activity to approximately 570.0 U/mL. Finally, the integrated process combining RY1 agitation, continuous feeding, and late-stage pH control at 8.0 produced 742.0 U/mL RML, 2.6-fold the control process.

The enhanced performance of RY1 was associated with a more favorable pellet morphology and oxygen-transfer environment, together with stage-dependent changes in intracellular amino-acid pools. In particular, the greater abundance of serine, threonine, and glutamine during the high-production stage was consistent with a precursor environment more favorable for RML synthesis. RQ nevertheless remained near 1.0, suggesting that the overall respiratory metabolic state was not substantially altered. Stable pellet architecture, a balanced morphology-oxygen-transfer relationship, sustained nutrient supply, and a well-timed late-stage pH trajectory therefore appear to have jointly supported high-level RML secretion. The resulting process provides a scalable framework for recombinant lipase production by filamentous fungi, although its reproducibility should be validated in larger reactors and the physiological effects of feeding and pH control warrant further investigation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Author contributions

Yuanyuan Jiang: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Yao Lan:** Writing – review & editing, Validation, Formal analysis. **Daolong Zhang:** Writing – review & editing, Data curation. **Ju Chu:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Feng Xu:** Supervision, Resources, Methodology. **Xiang Ke:** Writing – review & editing, Supervision, Resources, Methodology. **Xiwei Tian:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

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Figures

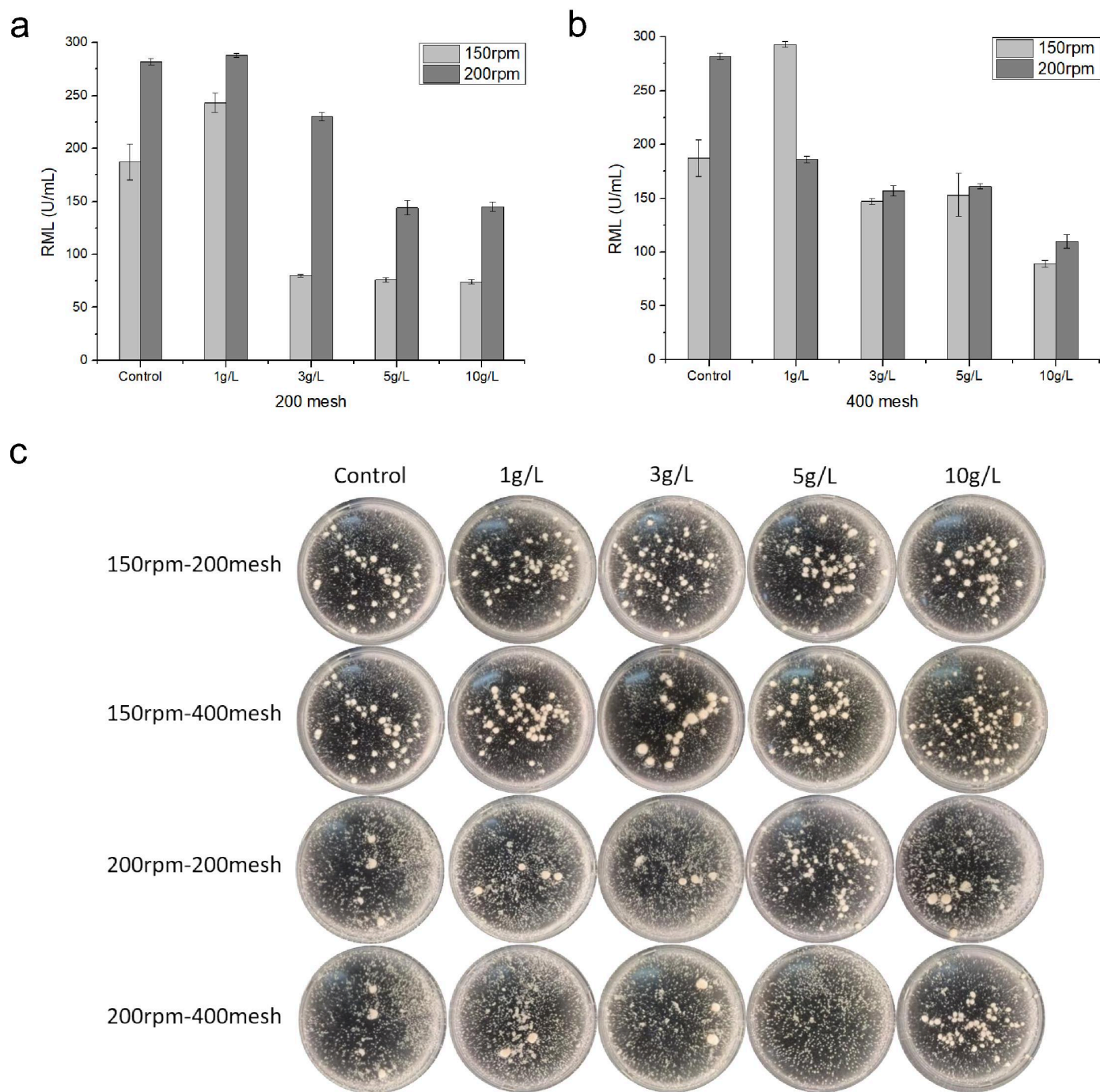


Figure 1

Effects of talc particle size and dosage on recombinant lipase activity and pellet morphology under different shaking speeds. (a) Enzyme activity obtained with 200-mesh talc at different dosages and rotation speeds. (b) Enzyme activity obtained with 400-mesh talc at different dosages and rotation speeds. (c) Macroscopic pellet morphology under different particle sizes, agitation speeds, and talc dosages. Data represent the mean $\pm$ standard deviation of biological triplicates.

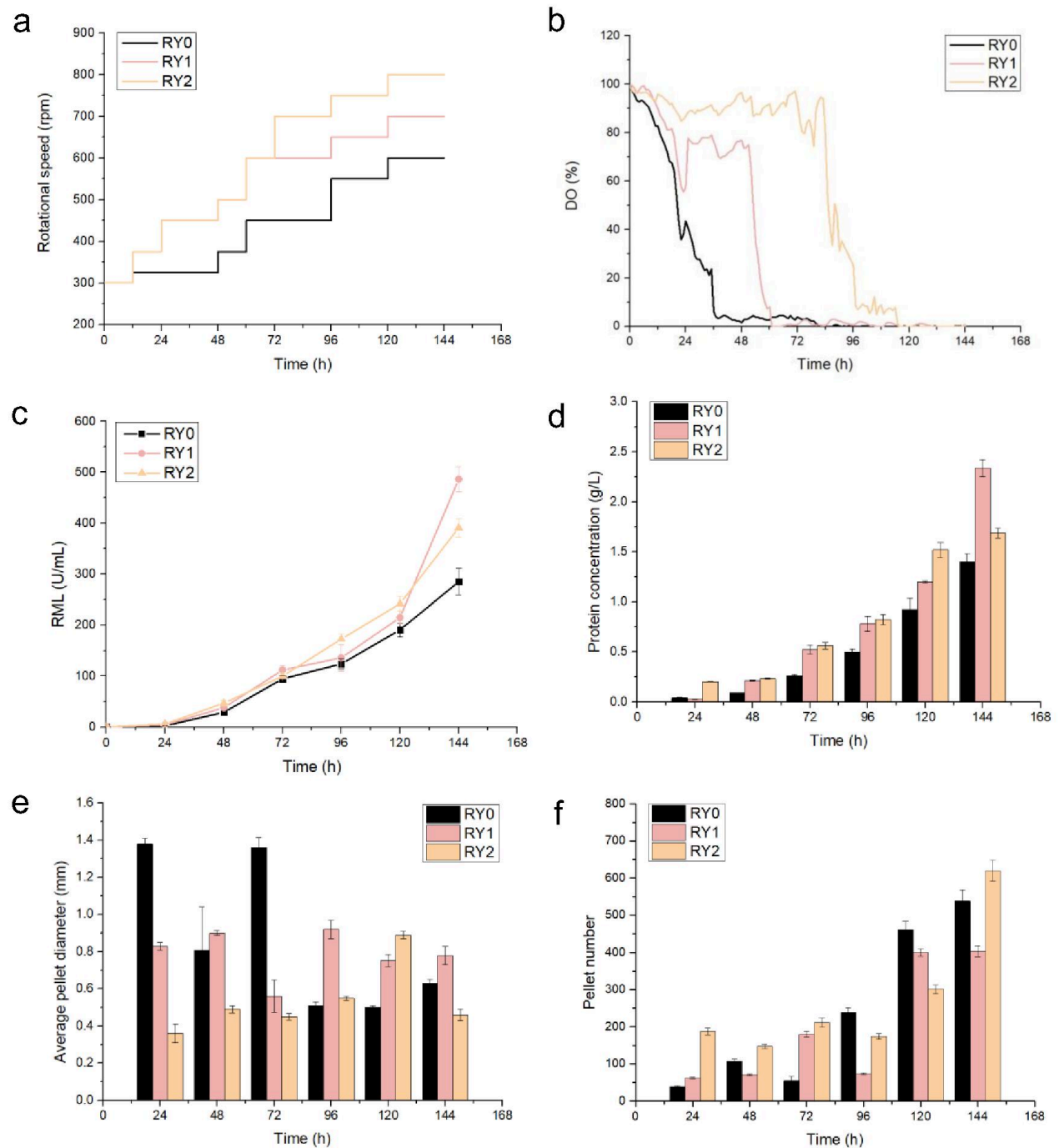


Figure 2

Effects of dynamic agitation strategies on recombinant lipase synthesis, dissolved oxygen, and pellet morphology during 5-L bioreactor fermentation. (a) Agitation trajectories for the original, dynamic-agitation, and intensified-dispersion processes. (b) Dissolved oxygen profiles. (c) Enzyme activity profiles. (d) Extracellular protein concentration profiles. (e) Changes in mean pellet diameter. (f) Changes

in pellet concentration. RY0, control process; RY1, optimized process; RY2, intensified-dispersion process. Data represent the mean $\pm$ standard deviation of biological triplicates.

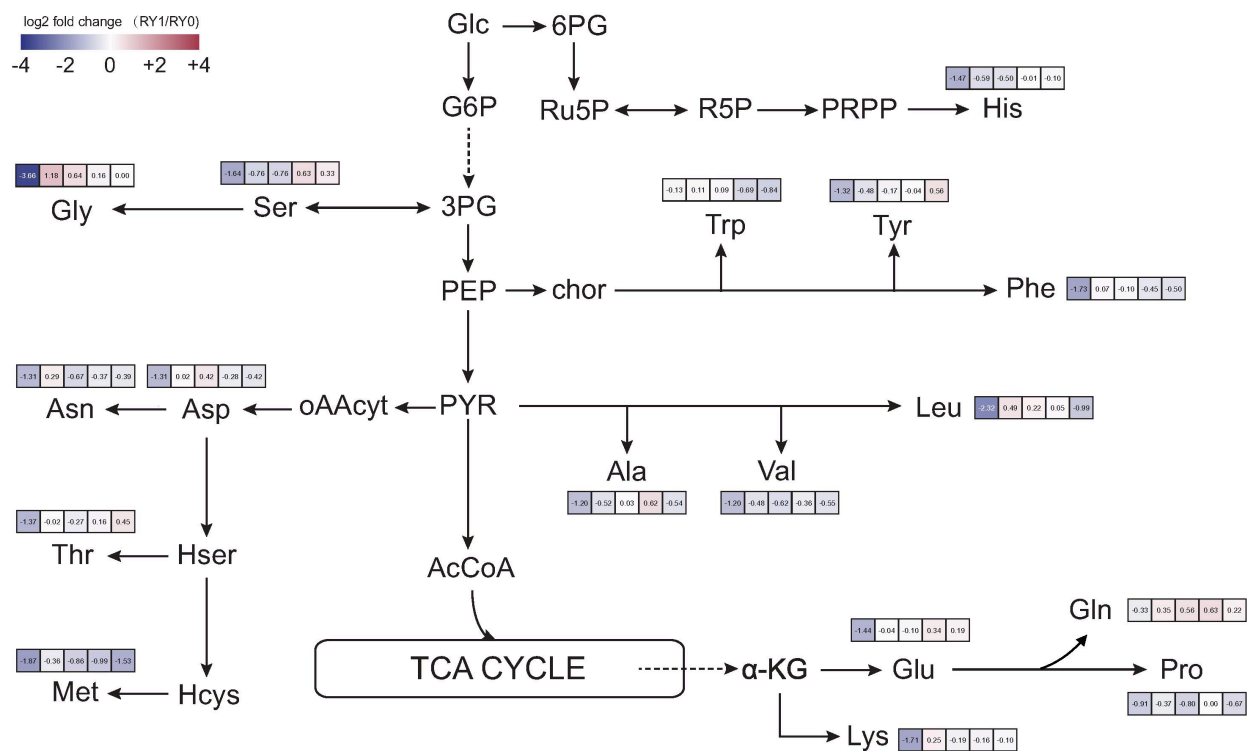


Figure 3

Relative changes in intracellular amino acid-related metabolites in optimized process (RY1) compared with control process (RY0). Each five-cell block shows the log₂(RY1/RY0) value at 24, 72, 96, 120, and 144 h, from left to right. Values were calculated from the mean normalized metabolite abundances obtained from three independent bioreactor runs for each process (n = 3). Solid arrows indicate direct metabolic reactions, whereas dashed arrows indicate multistep pathways. Standard three-letter amino acid abbreviations are used. Glc, glucose; 6PG, 6-phosphogluconate; G6P, glucose 6-phosphate; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; oAAcyt, cytosolic oxaloacetate; Hser, homoserine; Hcys, homocysteine; chor, chorismite; AcCoA, acetyl-CoA; α-KG, α-ketoglutarate; TCA, tricarboxylic acid.

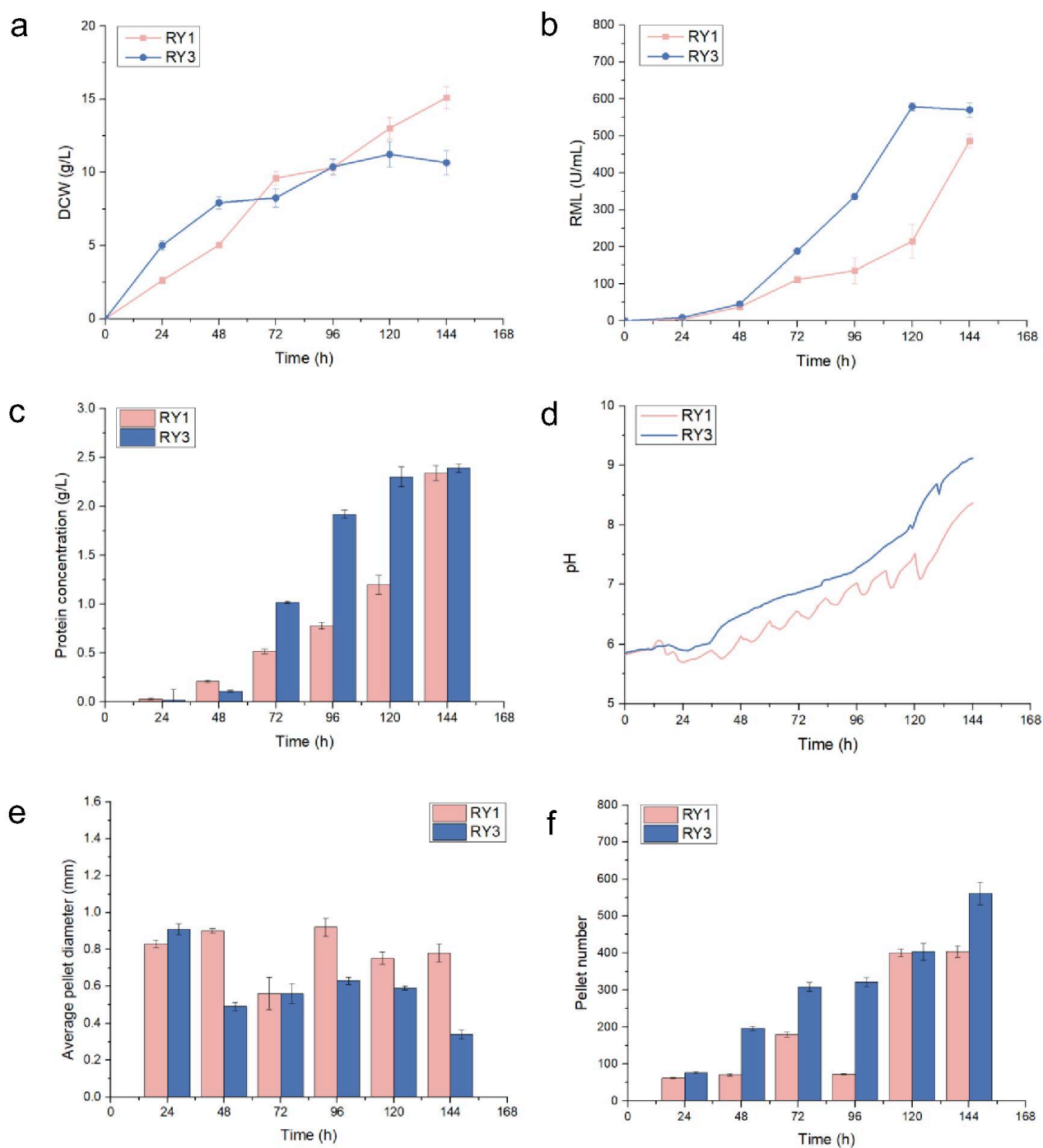


Figure 4

Effects of continuous feeding on fermentation performance and pellet evolution. (a) Dry cell weight (DCW). (b) RML activity. (c) Extracellular protein concentration. (d) Broth pH. (e) Mean pellet diameter. (f) Pellet concentration. Data represent the mean $\pm$ standard deviation of biological triplicates.

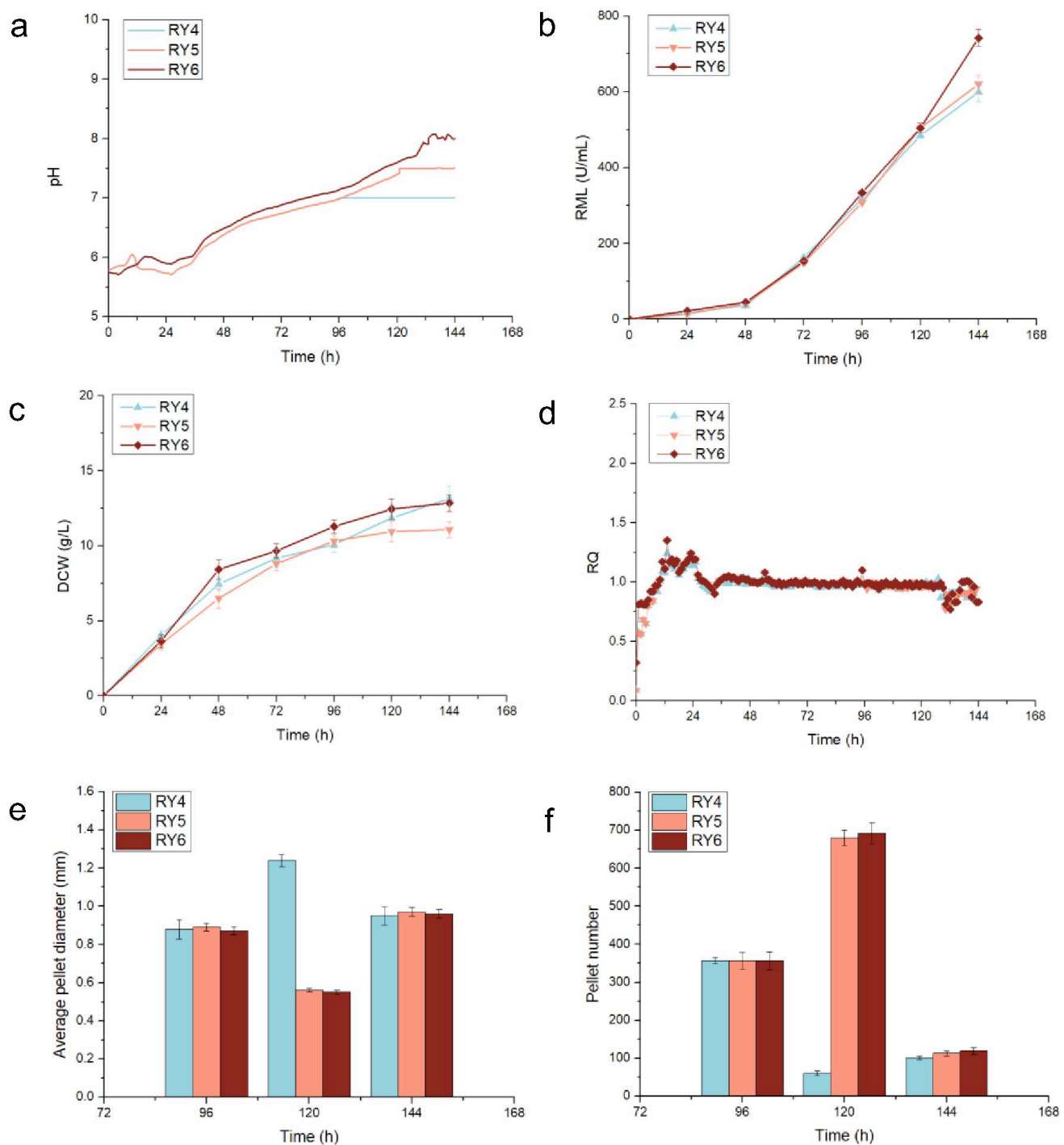


Figure 5

Effects of different late-stage pH-control strategies on RML production and pellet stability. (a) Broth-pH profiles. (b) RML activity. (c) Dry cell weight (DCW). (d) Respiratory quotient (RQ). (e) Mean pellet diameter at 96, 120, and 144 h. (f) Pellet concentration at 96, 120, and 144 h. RY4, pH allowed to increase naturally to 7.0 and then maintained at 7.0; RY5, pH allowed to increase naturally to 7.5 and

then maintained at 7.5; RY6, pH allowed to increase naturally to 8.0 and then maintained at 8.0. Data represent the mean $\pm$ standard deviation of biological triplicates.

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