Selection and Physiological Characterization of a High-Yield -Polylysine-Producing Strain with Dual Antibiotic Resistance: Postprint

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Abstract

Using Streptomyces albulus M-Z18, which exhibits an -polylysine titer of 1.60 g/L, as the parental strain, ribosome engineering technology was employed to select -polylysine high-yielding strains with dual antibiotic resistance, and the physiological and biochemical properties of the high-yielding strains were compared with those of the parental strain. Through streptomycin mutagenesis, a genetically stable -polylysine-producing strain, S. albulus S-7, was successfully obtained, exhibiting an -polylysine titer of 2.03 g/L; S. albulus S-7 was subsequently subjected to paromomycin selection, yielding a genetically stable -polylysine-producing strain with dual resistance, S. albulus SP-14, which achieved an -polylysine titer of 2.37 g/L, representing a 48.10% increase over the parental strain S. albulus M-Z18. The use of streptomycin and paromomycin for selecting -polylysine high-yielding strains with dual antibiotic resistance constitutes an effective strategy.

Full Text

Breeding and Physiological Characteristics of an -Polylysine High-Producing Strain with Dual Antibiotic Resistance

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Abstract

Using *Streptomyces albulus* M-Z18 with an -polylysine (-PL) yield of 1.60 g/L as the starting strain, we employed ribosomal engineering technology to breed high-yield -PL-producing mutants with dual antibiotic resistance and compared their physiological and biochemical properties with the parent strain. Through streptomycin mutagenesis, we successfully isolated a genetically stable -PL-producing strain *S. albulus* S-7 with a yield of 2.03 g/L. By subsequently applying paromomycin selection to S-7, we obtained a genetically stable double-resistant -PL-producing strain *S. albulus* SP-14 with a yield of 2.37 g/L, representing a 48.10% increase compared to the original *S. albulus* M-Z18. These results demonstrate that using streptomycin and paromomycin to screen for double antibiotic-resistant -PL high-producing strains is an effective approach.

Keywords: fermentation engineering; physiological and biochemical properties; dual antibiotic resistance; -polylysine

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-Poly-L-lysine (-PL) consists of 25-35 L-lysine (L-Lys) monomers linked by amide bonds between -COOH and -NH groups. As a novel food preservative, -PL possesses unique properties including non-toxicity and broad-spectrum antimicrobial activity, effectively inhibiting the growth of Gram-positive bacteria, yeasts, and fungi [1]. Additionally, -PL is environmentally friendly and widely used in the biomedical industry. With its broad application range and substantial market value, -PL represents a promising new biotechnology product.

Various wild-type strains produce -PL, such as *Streptomyces noursei* NRRL 5126 and *Streptomyces griseofuscus* H1, but their production capacities are low at 0.42 g/L and 0.70 g/L, respectively [2-4], making them unsuitable for industrial applications. Strain improvement is essential to enhance target metabolite production for industrialization. In recent years, numerous physical and chemical mutagenesis methods have been applied to improve -PL-producing strains. For example, diethyl sulfate mutagenesis of *Kitasatospora* sp. PL6-3 [5] followed by screening for AEC resistance yielded mutant strain MY5-36 with an -PL yield of 1.17 g/L, three times higher than the parent strain. Other researchers have employed atmospheric and room temperature plasma (ARTP) mutagenesis and genome shuffling techniques. In 2012, Zong et al. [6] increased the -PL yield of *S. albulus* A-29 from 0.40 g/L to 1.59 g/L through ARTP mutagenesis. While physical and chemical methods can effectively improve -PL yields, these traditional breeding approaches are time-consuming, labor-intensive, and inefficient.

In 2007, Japanese scholar Ochi et al. proposed a novel mutagenesis method called ribosomal engineering, which involves constructing resistant strain libraries and selecting ribosomal mutants to obtain strains with improved target metabolite production. In 1996, Ochi et al. [7] introduced streptomycin resistance into *Streptomyces lividans* and *Streptomyces coelicolor* A3(2), significantly increas-

ing actinorhodin production. In 2009, Wang et al. [8] introduced paromomycin resistance into *S. coelicolor*, substantially enhancing the production of undecylprodigiosin and actinorhodin. These findings demonstrate that ribosomal engineering is an effective method for improving secondary metabolite production in *Streptomyces*. However, no studies have reported the simultaneous introduction of both streptomycin and paromomycin resistance into -PL-producing strains.

This study used a *Streptomyces albulus* M-Z18 strain as the starting strain to improve antibiotic tolerance and -PL production by introducing streptomycin and paromomycin resistance, and compared the physiological and biochemical properties of the high-yield mutant with the parent strain.

Materials and Methods

1.1 Strain The -PL-producing strain *S. albulus* M-Z18 was domesticated and preserved in our laboratory.

1.2 Media **Solid medium (BTN, g/L):** glucose 10, peptone 2, yeast extract 1, agar 20, pH 7.50.

Seed medium (M3G, g/L): glucose 50, yeast extract 5, ammonium sulfate 10, KH PO 1.36, K HPO 0.80, MgSO · 7H O 0.50, ZnSO · 7H O 0.04, FeSO · 7H O 0.03, pH 6.80.

Optimized medium (YP/YG, g/L): glucose 30/glycerol 60, yeast extract 8, ammonium sulfate 5, MgSO · 7H O 0.50, KH PO 2, ZnSO · 7H O 0.04, FeSO · 7H O 0.03, pH 7.50. Abbreviated as YP when using glucose as carbon source and YG when using glycerol.

RSM medium (g/L): glycerol 60, yeast extract 10, ammonium sulfate 5, MgSO · 7H O 0.80, KH PO 4, FeSO · 7H O 0.05, pH 6.80.

All media were sterilized at 115 °C for 15 min.

1.2.1 Preparation of Single Spore Suspension A glycerol stock stored at -80 °C was thawed and spread onto solid plates, then incubated at 30 °C for 8 days. Single spore suspensions were prepared according to reference [9]. Spores were washed from solid plates with sterile water, collected, vortexed, and filtered through gauze to obtain single spore pellets. The pellets were washed with sterile water until no impurities remained, and the spore concentration was adjusted to 10 CFU · mL [1].

1.2.2 Determination of Minimum Inhibitory Concentration (MIC) Spore suspensions of *S. albulus* M-Z18 were appropriately diluted to approximately 10 CFU · mL [1] and spread onto antibiotic-containing plates, with three replicates per concentration. Colony growth was observed after incubation at

30 °C for 6 days, and the critical concentration showing no colony growth was recorded as the MIC.

1.2.3 Screening for Streptomycin-Resistant -PL High-Producing Strains Spores were spread onto plates containing 7-10 mg/L streptomycin and incubated at 30 °C for 8-10 days. Single colonies with larger diameters and dense spores were selected for expansion culture. After 8 days of incubation at 30 °C, primary screening was performed using a high-throughput system [10]. The positive mutation rate (RP) and mutation rate (RM) were calculated: strains with >5% higher yield than the parent were recorded as positive mutants (P), while those with >5% lower yield were recorded as negative mutants (N). Positive mutants from primary screening were verified through shake-flask rescreening to confirm fermentation performance, with RP and RM calculated using the same method.

The total number of mutants (M), positive mutation rate (RP), and mutation rate (RM) were calculated as follows: %100 × (M/T) = RM %100 × (P/T) = RP

Where T is the total number of selected single colonies, M is the total number of mutant strains, P is the number of positive mutants, and N is the number of negative mutants.

1.2.4 Screening for Double-Resistant -PL High-Producing Strains Spore suspensions of S-7 were appropriately diluted and spread onto plates containing paromomycin, then incubated at 30 °C for 8-10 days. Single colonies with larger diameters and abundant spores were selected for expansion culture. After 8 days of incubation at 30 °C, primary screening was performed using the high-throughput system followed by shake-flask rescreening. The top three double-resistant -PL mutants from rescreening were preserved.

1.2.5 Primary and Secondary Screening **Primary screening:** Appropriate spores were scraped with a toothpick and inoculated into 24-deep-well plates containing 2 mL YG medium. Cultivation conditions: 30 °C, 200 r/min for 96 h. -PL concentration was determined by the methyl orange method [11].

Rescreening: Spores were inoculated into YG medium and cultured for 24 h. Seed culture was transferred to 250 mL shake flasks containing 40 mL YG medium and cultured for 96 h. -PL concentration was measured to select high-yield strains.

1.3.1 Mycelial Morphology on Solid Medium Spore suspensions of M-Z18 and SP-14 were spread onto solid medium and incubated at 30 °C until white mycelia appeared. Mycelial morphology was observed under scanning electron microscopy.

1.3.2 Spore Morphology on Solid Medium Spore suspensions of M-Z18 and SP-14 were spread onto solid medium and incubated at 30 °C until spores formed. Spore morphology was observed under scanning electron microscopy.

1.3.3 Pellet Morphology in Liquid Medium Spores of M-Z18 and SP-14 were cultured in YG medium for 24 h, then seed culture was transferred to fresh YG medium. Multiple sampling points were set to observe pellet formation and lysis. Pellet morphology was examined under a light microscope.

1.3.4 Antibiotic Resistance Capability Spores of M-Z18 and SP-14 were appropriately diluted to approximately 10 CFU · mL ^{1} and spread onto antibiotic plates, then incubated at 30 °C. Colony growth was observed.

1.3.5 Effects of Different Media on -PL Shake-Flask Fermentation Yield Spores of M-Z18 and SP-14 were cultured for 4 days in four different media (M3G, YG, YP, and RSM) with three replicates each to compare -PL yields between the high-yield mutant and parent strain.

1.3.6 Comparison of Natural Fermentation Processes in Shake Flasks Spores of M-Z18 and SP-14 were inoculated into YG medium and cultured at 30 °C, 200 r · min ^{1} for 1 day, then transferred to YG medium for 3 days of fermentation. Parameters including pH, glycerol consumption, dry cell weight, and -PL concentration were analyzed and compared.

-PL Metabolic Pathway Key Enzyme Activity Assays Spores of M-Z18 and SP-14 were inoculated into YG medium. After 48 h, broth was centrifuged at 4500×g for 15 min to collect cells. Cells were washed with 0.20% KCl and centrifuged at 4500×g for 15 min (repeated twice), then suspended in 100 mmol/L Tris-HCl (pH 7.50) at a ratio of 5 mL buffer per 1 g cells. Cells were disrupted by ultrasonication and centrifuged at 12000×g for 20 min; the supernatant served as crude enzyme extract. Enzyme activity assays for key enzymes in the pentose phosphate pathway (PPP) [glucose-6-phosphate dehydrogenase (G6PDH)], glycolysis (EMP) and TCA cycle [pyruvate kinase (PK) and citrate synthase (CS)], anaplerotic pathway [phosphoenolpyruvate carboxylase (PEPC) and pyruvate carboxylase (PYC)], diaminopimelic acid (DAP) pathway [aspartokinase (ASK)], and -PL synthesis pathway [-PL synthetase] were performed according to reference [12].

Results

2.1 Minimum Inhibitory Concentration (MIC) of Antibiotics Figure 1 Figure 1: see original paper shows the colony growth of *S. albulus* M-Z18 on solid plates containing different streptomycin concentrations. On antibiotic-free

plates (Figure 1(1)A), dense spores formed after 6 days. At 2 mg/L streptomycin (Figure 1(1)B), sparse colonies with light-colored spores appeared, indicating antibiotic inhibition. As concentration increased, colony numbers decreased progressively with no spore formation. At 6 mg/L streptomycin (Figure 1(1)D), growth was completely inhibited, establishing the MIC of streptomycin for M-Z18 as 6 mg/L. Using the same method for paromomycin, Figure 1(2) shows M-Z18 growth on plates with different paromomycin concentrations. At 6 mg/L paromomycin (Figure 1(2)B), growth was significantly inhibited with few colonies and no spore formation compared to the control (Figure 1(2)A). At 9 mg/L (Figure 1(2)C), virtually no single colonies existed, establishing the MIC of paromomycin for M-Z18 as 9 mg/L.

Figure 1 Growth of *S. albulus* M-Z18 on solid plates containing different concentrations of streptomycin and paromomycin on day 6. Note: (1) A: 0 mg/L; B: 2 mg/L; C: 4 mg/L; D: 6 mg/L; (2) A: 0 mg/L; B: 6 mg/L; C: 9 mg/L; D: 10 mg/L.

2.2 Screening for Streptomycin-Resistant -PL High-Producing Strains *S. albulus* M-Z18 spores were spread onto streptomycin-containing plates and incubated at 30 °C for 8 days, yielding 112 isolates for the mutant library. High-throughput primary screening and shake-flask rescreening identified 23 strains with improved production, achieving a positive mutation rate of 54.76% (Table 2). The average shake-flask yield was 1.95 ± 0.10 g/L, a 21.88% increase over the parent strain *S. albulus* M-Z18. One mutant strain, S-7, was obtained with a yield of 2.03 ± 0.10 g/L (Table 1).

Statistical analysis of primary and secondary screening results (Table 2) revealed that streptomycin resistance screening achieved a mutation rate of 40.18% (considering strains with >5% yield change as mutants) and a positive mutation rate of 57.78% in primary screening. Rescreening of primary positive mutants confirmed high fermentation performance, maintaining a positive mutation rate of 54.76%. This is approximately 20% higher than the maximum positive mutation rate of 32% achieved through ARTP mutagenesis of *Streptoverticillium mobaraense* [13], indicating that ribosomal engineering via streptomycin resistance is more efficient for breeding -PL high-producing strains in *S. albulus* M-Z18.

Streptomycin resistance mutations primarily occur in ribosomal protein S12, such as K88R and R86H mutations [14]. To understand the high-yield mechanism, researchers investigated ribosomes in high-producing mutants and found significantly improved stability of the 70S ribosomal complex under low magnesium concentrations, along with elevated expression of ribosome recycling factor (RRF), which is crucial for ribosome-mRNA-tRNA termination complex dissociation. The increased RRF level led to abnormally enhanced protein synthesis activity during the late stationary phase, resulting in overproduction of target metabolites [15-16].

2.3 Screening for Double-Resistant -PL High-Producing Strains S-7 spores were spread onto solid medium containing 9-36 mg/L paromomycin and incubated at 30 °C for 8 days. However, single colonies showed only mycelial growth without spores or even failed to grow, indicating excessive antibiotic concentration that severely inhibited strain growth. Resistance plate concentrations should both inhibit growth and allow survival of better-growing colonies [17]. Therefore, paromomycin concentration was adjusted. As shown in Figure 2 [Figure 2: see original paper], strain S-7 grew well with dense spores on antibiotic-free plates (A). With increasing antibiotic concentration, colony numbers decreased while spore formation ability diminished. At 6.3 mg/L (E), some colonies produced spores, but at 8.1 mg/L (F), no spores formed. Genetic stability is a crucial trait for industrial microorganisms and should be prioritized during strain breeding. Based on Figure 2, screening concentrations of 4.5 mg/L and 6.3 mg/L paromomycin were selected.

Figure 2 Growth of *S. albulus* S-7 on solid plates containing different paromomycin concentrations on day 6. Note: A: 0 mg/L; B: 0.9 mg/L; C: 2.7 mg/L; D: 4.5 mg/L; E: 6.3 mg/L; F: 8.1 mg/L.

A total of 61 single colonies were selected from resistance plates. After high-throughput primary screening and shake-flask rescreening, 6 strains showed improved -PL production with a positive mutation rate of 31.58% (Table 2). The average shake-flask yield was 2.30 ± 0.10 g/L, a 13.30% increase over parent strain S-7. One mutant strain, SP-14, was obtained with a yield of 2.37 ± 0.10 g/L (Table 1).

The positive mutation rate for double-resistant (streptomycin and paromomycin) strains (31.58%) was lower than that for single-resistant (streptomycin) strains (54.76%), suggesting that while dual resistance can further improve -PL yield, the efficiency of yield improvement may decrease with increasing resistance. However, compared to the 16.67% positive mutation rate achieved through chemical mutagenesis [18], the positive mutation rate obtained by stacking antibiotic resistances remains substantially higher.

Wang et al. [19] found that paromomycin resistance can cause mutations in the *rpsL* gene, inserting a glycine residue at position 92 of ribosomal protein S12 in *S. coelicolor*. This insertion mutation (GI92) increased paromomycin resistance 20-fold and enhanced antibiotic overproduction, demonstrating that paromomycin addition can be used to improve target product yields.

Table 1 Statistical data of screening drug-resistant mutants

Experiment	Parent strain (yield)	Screening antibiotic concentration (mg/L)	Average yield (g · L ^{1})	Highest yield (g · L ^{1})
Streptomycin-resistant screening	M-Z18 (1.60 g/L)	Streptomycin: 7-10	1.95 ± 0.10	2.03 ± 0.10
Double-resistant screening	S-7 (2.03 g/L)	Paromomycin: 4.5-6.3	2.30 ± 0.10	2.37 ± 0.10

Table 2 Statistical data of mutation results

Screening stage	Mutants (M)	Positive mutants (P)	Total colonies (T)	Mutation rate (RM)	Positive mutation rate (RP)
Streptomycin primary	-	-	-	40.18%	57.78%
Streptomycin rescreening	23	-	42	-	54.76%
Double resistance primary	-	-	-	-	-
Double resistance rescreening	6	-	19	-	31.58%

2.4.1 Mycelial and Spore Morphology Using *S. albulus* M-Z18 as the starting strain, we obtained the double-resistant mutant SP-14. Mycelial and spore morphologies of both strains are shown in Figure 3 [Figure 3: see original paper]. M-Z18 (A-1) mycelia exhibited numerous small, round vesicles on their surface, while SP-14 (B-1) showed many small white granular substances that gradually developed into vesicles only after spore formation. This difference reflects the reduced growth rate of SP-14 after mutagenesis; within the same growth period, obvious vesicles had not yet formed on SP-14 mycelia until spore formation occurred. Additionally, SP-14 mycelia appeared shriveled compared to M-Z18.

Comparing spore morphology, M-Z18 (A-2) spores had few and small surface spines, while SP-14 (B-2) spores exhibited uniformly distributed spines with sharp, needle-like projections [20]. Furthermore, spore density differed, with M-Z18 producing dense spores while SP-14 (B-2) showed reduced spore numbers, indicating diminished sporulation ability after mutagenesis.

Figure 3 Comparison of mycelium and spore morphology of *S. albulus* M-Z18 (A) and SP-14 (B) on solid plates.

2.4.2 Pellet Morphology Differences To further understand the different growth states during fermentation, we observed pellet morphology of M-Z18 and SP-14 in YG medium at different time points, as pellet morphology affects oxygen and nutrient acquisition.

As shown in Figure 4 [Figure 4: see original paper], SP-14 appeared linear at 24 h while M-Z18 had already formed pellets, indicating slower growth of SP-14. After 48 h, both strains showed distinct pellet morphologies: SP-14 pellets were slightly smaller than M-Z18 with smaller, hollow centers, while M-Z18 pellet centers were dark, suggesting growth inhibition in the mutant. At 96 h (Figure 4 A-96), M-Z18 showed lysis with many broken hyphae and incomplete pellet structure, whereas SP-14 (B-96) maintained relatively intact pellet morphology with only reduced central volume compared to 48 h. The slower growth of SP-14 correlated with its reduced spore numbers and sporulation ability under the same conditions (Figure 3).

Researchers have investigated how different carbon/nitrogen sources and ratios affect pellet morphology. The results showed that pellet morphology differences affect fermentation broth rheology, which influences nutrient and oxygen transfer, subsequently affecting metabolism and final product synthesis [21]. Moreover, maintaining a certain pellet core volume during mid-to-late fermentation is beneficial for product synthesis. SP-14' s advantage lies in maintaining relatively intact pellet morphology during the later stages, enabling sustained fermentation capacity.

Figure 4 Comparison of pellet morphology of *S. albulus* M-Z18 (A) and SP-14 (B) at different fermentation time points in shake flasks (100×).

2.4.3 Antibiotic Resistance Capability Ribosomal engineering of *S. albulus* M-Z18 yielded strains with streptomycin resistance and combined streptomycin-paromomycin resistance. These mutants showed enhanced -PL synthesis capacity and altered tolerance to other antibiotics, a phenomenon confirmed by many studies. We measured antibiotic tolerance of *S. albulus* M-Z18, S-7, SP-14, and other resistant strains used in this study by observing growth on antibiotic plates at various concentrations.

Table 3 reveals: (1) Actual resistance levels were higher than the antibiotic concentrations used for screening, as resistance mutations further enhanced antibiotic tolerance; (2) During paromomycin resistance screening, paromomycin

showed strong growth inhibition. When using the same screening strategy as streptomycin, no colonies or spores formed on paromomycin plates, necessitating the modified screening approach shown in Figure 2. Although mutants were obtained at lower screening concentrations, their actual resistance changed substantially; (3) After stacking streptomycin resistance, tolerance to paromomycin also increased, and vice versa, indicating that mutagenesis enhanced resistance to different antibiotics simultaneously.

Table 3 Drug-resistance of strains used in this study

Strain	Screening antibiotic concentration (mg · L ^{1})	-PL yield (g · L ^{1})	Resistance (mg · L ^{1})
M-Z18	6.00 (streptomycin)	1.60	6.00 (streptomycin)
SP-14	6.00 (streptomycin)	2.37	10.50 (paromomycin)
M-Z18	6.00 (streptomycin)	1.60	7.50 (paromomycin)

2.4.4 Effects of Different Media on -PL Fermentation Yield Genetically engineered high-yield strains often exhibit altered nutritional requirements due to significantly increased target metabolite production. We compared -PL yields of M-Z18 and SP-14 in four laboratory media (M3G, RSM, YG, and YP) to investigate changes in nutritional requirements.

Figure 5 [Figure 5: see original paper] shows that SP-14 exhibited different -PL yield rankings across media (YG > RSM > YP > M3G) compared to M-Z18 (RSM > YG > YP > M3G). While M-Z18 achieved maximum yield in RSM medium (1.59 g/L), SP-14 produced highest yields in YG medium (2.32 g/L). This suggests that after two rounds of breeding, SP-14' s -PL synthesis pathway differed substantially from the parent strain. SP-14 produced higher -PL yields than M-Z18 in all media, indicating strengthened -PL synthesis capacity.

Figure 5 Comparison of -PL production by *S. albulus* M-Z18 and SP-14 in different media.

2.4.5 Comparison of Natural Fermentation Processes in Shake Flasks *S. albulus* M-Z18 produces approximately 1.60 g/L -PL in shake flasks with stable genetic characteristics. Using this superior strain as the foundation, we obtained *S. albulus* SP-14 with -PL yield reaching 2.37 g/L through ribosomal engineering. To better understand the differences between M-Z18 and SP-14, we conducted natural fermentation in YG medium, comparing parameters including pH, glycerol consumption, dry cell weight, and -PL concentration.

The results (Figure 6 [Figure 6: see original paper]) showed similar trends for all measured parameters. pH dropped sharply from 7.5 to approximately 4.0

within 20-40 h, then decreased slowly, with no significant difference between SP-14 and M-Z18. Glycerol consumption patterns were similar, though slightly faster in M-Z18, likely due to growth impairment in SP-14 after multiple rounds of mutagenesis. Dry cell weight of M-Z18 (peaking at 6.26 g/L at 66 h) was higher than SP-14. No product was detected at 24 h, but -PL became detectable at 30 h. The synthesis rate of SP-14 remained consistently higher than M-Z18, possibly due to altered -PL synthesis pathways and enhanced synthetic capacity in SP-14.

Figure 6 Shake-flask fermentation comparison between *S. albulus* M-Z18 and SP-14.

2.4.6 Key Enzyme Activity Analysis in -PL Synthesis-Related Central Carbon Metabolism After antibiotic mutagenesis of M-Z18, high-yield strain SP-14 was obtained with 48.10% increased -PL production. To investigate the high-yield mechanism at the physiological and metabolic level, we examined key enzyme activities in core carbon metabolism pathways related to -PL synthesis.

The pentose phosphate pathway (PPP) provides pentose sugars and NADPH for cell growth and metabolite synthesis, with G6PDH as the first rate-limiting enzyme. Figure 7 [Figure 7: see original paper]A shows that SP-14 exhibited higher G6PDH activity than M-Z18, indicating enhanced pentose synthesis beneficial for cell growth. Therefore, strengthening PPP key enzyme activity through strain modification could improve metabolic flux through this pathway.

The EMP pathway produces pyruvate, the carbon skeleton required for the TCA cycle. PK activity directly affects the flux of these carbon skeletons into the TCA cycle. As shown in Figure 7A, SP-14 showed approximately 2-fold higher PK activity than M-Z18, suggesting that enhancing EMP pathway metabolism could provide more carbon skeletons for the TCA cycle. The TCA cycle synthesizes NADH and FADH while providing precursors for other metabolites, such as oxaloacetate required for L-Lys synthesis. Figure 7A shows SP-14 had approximately 1.70-fold higher CS activity than M-Z18, facilitating greater NADH synthesis for respiratory energy production.

Oxaloacetate is the carbon skeleton precursor for L-Lys synthesis. Since large amounts of L-Lys are consumed during -PL synthesis, intracellular oxaloacetate becomes relatively scarce. However, the TCA cycle does not net-produce oxaloacetate, requiring cells to replenish it through PEPC and PYC. Figure 7A reveals that SP-14 showed increased activities of both PEPC and PYC compared to M-Z18, indicating stronger oxaloacetate replenishment capacity beneficial for L-Lys precursor synthesis.

In bacteria, L-Lys is synthesized from L-aspartate (L-Asp) via the DAP pathway, which begins with L-Asp phosphorylation catalyzed by ASK. As the key enzyme in the DAP pathway, ASK directly affects L-Lys supply capacity. Figure 7A shows SP-14 exhibited 2-fold higher ASK activity, suggesting strong

L-Lys synthetic metabolism to supply abundant L-Lys for -PL synthesis. To verify this, intracellular L-Lys content was measured. Since -PL is polymerized from L-Lys, -PL synthetase activity was defined by L-Lys consumption per unit time. Figure 7B shows Pls enzyme activity in M-Z18 and SP-14. The high-yield mutant SP-14 showed significantly higher Pls activity than M-Z18, confirming that enhanced ASK activity provides abundant L-Lys for -PL synthesis.

Figure 7 Enzymatic activity comparison of *S. albulus* M-Z18 and SP-14 during shake-flask fermentation.

Conclusion

This study employed ribosomal engineering for strain improvement using streptomycin and paromomycin resistance screening to obtain mutant strains *S. albulus* S-7 and SP-14 with -PL yields of 2.03 g/L and 2.37 g/L, respectively, representing 26.90% and 48.10% increases over the parent strain M-Z18. Stacking paromomycin resistance onto the single-resistant S-7 strain yielded double-resistant mutant SP-14 with significantly enhanced -PL production, likely due to combined effects of mutations introduced by both antibiotics. The mutant SP-14 accumulated higher levels of ribosome recycling factor during late stationary phase than S-7, providing the foundation for high-level protein synthesis activity. These results demonstrate the effectiveness of introducing multiple resistance mutations for improving metabolite production.

Physiological and biochemical characterization revealed altered mycelial, spore, and pellet morphologies in the mutants. Testing -PL yields in different media showed changed nutritional requirements, with optimized medium (YG) being most favorable for -PL production. Analysis of key enzymes related to cell growth and -PL synthesis showed increased activities of G6PDH, PK, PEPC, CS, PYC, ASK, and Pls in the high-yield strain compared to the parent. These findings indicate that improving antibiotic tolerance and stacking antibiotic resistances can enhance metabolic capacity related to -PL synthesis in *S. albulus*.

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Note: Figure translations are in progress. See original paper for figures.