Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) in press
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Correspondence

https://doi.org/10.1631/jzus.B2400477

Improvement of neutral protease activity of *Bacillus amyloliquefaciens* LX-6 by combined ribosome engineering and medium optimization and its application in soybean meal fermentation

Yifan ZHU[1*], Xinyi HUANG[1*], Tao HAN[2✉], Jiteng WANG[2], Xiaoping YU[1], Zheng MA[1✉]

[1]*Key Laboratory of Microbiological Metrology, Measurement & Bio-product Quality Security, State Administration for Market Regulation, College of Life Sciences, China Jiliang University, Hangzhou 310018, China*
[2]*Department of Aquaculture, Zhejiang Ocean University, Zhoushan 316000, China*

Unedited

Soybean meal (SBM) prepared by soybean crushing is the most popular protein source in the poultry and livestock industries (Cai et al., 2015) due to its economic manufacture, high protein content and good nutritional value. Despite these benefits, SBM contains various antigen proteins such as glycinin and β-conglycinin, which account for approximately 70% of total protein of the SBM and reduce digestibility and damage intestinal function (Peng et al., 2018). Treating SBM with proteases (neutrase, alcalase and trypsin) or fermentation can eliminate these antigen proteins (Contesini et al., 2018). Because of its safety and rapid growth cycle, *Bacillus* strains are considered ideal for the fermentation industry (Yao et al., 2021). SBM fermented by *Bacillus* yields products with high nutritional value and low levels of antinutritional factors (ANF), stimulating research in this area (Yuan et al., 2017). Kumari et al. (2023) demonstrated that fermentation with *Bacillus* species effectively degrades antigen proteins and increases crude protein content. The degradation of antigen proteins relies on protease hydrolysis. Currently, low protease production is the major obstacle hindering the widespread use of microbial fermentation techniques.

Ribosome engineering is an approach that introduces spontaneous mutations into the ribosome during drug-resistant microorganisms (Wang et al., 2020). Recent studies have shown that various enzymes in *Bacillus* can be significantly enhanced by introducing such mutations that confer resistance to rifamycin (Rif) and streptomycin (Str) (Suzuki et al., 2018). In Str-resistant (Str^r) mutants, mutations occur in the *rpsL* gene that encodes the ribosomal S12 protein, whereas in Rif-resistant (Rif^r) mutants, the mutations are found in the *rpoB* gene (Zhu et al., 2019). Kurosawa et al. (2006) demonstrated that introducing mutations conferring antibiotic resistance enhances α-amylase and protease production in *Bacillus subtilis*, as measured by enzyme activity. Similarly, Nie et al. (2021) improved the keratinase synthesis production of *Bacillus thuringiensis* by inducing Str resistance.

✉ Zheng MA, mazheng520@163.com
Tao HAN, goodhantao@gail.com
* The two authors contributed equally to this work
Zheng MA, https://orcid.org/0000-0002-1446-0708
Tao HAN, https://orcid.org/0000-0003-1269-5834
Yifan ZHU, https://orcid.org/0009-0000-4003-3315

Received Sept. 19, 2024; Revision accepted Dec. 26, 2024;
Crosschecked xxx. xx, 20xx; Published online xxx. xx, 20xx

© Zhejiang University Press 2025

Protease is primarily produced through microbial fermentation; however, industrial production and application has been limited due to the associated high cost and low productivity (Song et al., 2023). The synthesis of protease is strongly influenced by medium composition, including carbon and nitrogen sources and metal ion species (Zhou et al., 2023). The medium components can be optimized by using conventional methods that study one variable at a time while keeping others fixed (Koim-Puchowska et al., 2021), making the process tedious and time-consuming. In contrast, response surface methodology (RSM) allows for the simultaneous evaluation of multiple variables and their interactions, offering a fast and reliable approach (Homayoonfal et al., 2024; Zhang et al.,2020).

In our previous study, *Bacillus amyloliquefaciens* LX-6 (China Center for Type Culture Collection (CCTCC) M 20211457), a neutral protease producer, was isolated and identified from soil samples and the strain's neutral protease activity 6 was measured at 1390 U/mL (Huang et al., 2023). Given this protease production capability, strain LX-6 shows significant potential for SBM fermentation, although its current protease yield is insufficient for industrial production. In this study, we employed ribosome engineering to enhance the neutral protease production of strain LX-6 by inducing its Rif resistance. Then, we applied single-factor optimization experiments and response surface methodology (RSM) to optimize the media components, aiming to further enhance the neutral protease activity in shake flask experiments. Finally, we selected the Rif^r mutant strain with the highest neutral protease activity for SBM fermentation and evaluated the degradation of glycinin and β-conglycinin.

The MIC of Rif against *B. amyloliquefaciens* LX-6 was determined to be 5 μg/mL. LB agar plates supplemented with Rif concentrations of 5 μg/mL and 10 μg/mL were employed to isolate the Rif^r mutants of *B. amyloliquefaciens* LX-6, and a total of 253 spontaneous Rif^r mutants were isolated at 5 μg/mL Rif concentration. Among them (Table 1), six exhibited larger hydrolysis zones on skim milk agar plates compared to the parental strain *B. amyloliquefaciens* LX-6, while two mutants showed significantly reduced hydrolysis zones (Table2). Additionally, 53 spontaneous Rif^r mutants were isolated at a Rif concentration of 10 μg/mL; however, none displayed increased hydrolysis zones comparable to the parental strain *B. amyloliquefaciens* LX-6.

Unedited

Table 1. Summary of the isolation mutants of *B. amyloliquefaciens* LX-6

Antibiotics	Concentration of antibiotics used for selection of mutants (μg/mL)[a]	Frequency (%) of mutants exhibiting bigger hydrolysis zone diameters	Biggest hydrolysis zone diameters (cm)[b]
Rifamycin	5	0.24 (6/253)	2.4
	10	0	NF[c]

a: The MIC of Rif for WT strain *B. amyloliquefaciens* LX-6 was 5 μg/mL
b: The hydrolysis zone diameter of the WT strain LX-6 was 2.1 cm
c: Not found

Table 2. Analyses of the neutral protease production and mutation positions in representative Rif^r mutants

Strains	Average neutral protease production (U/mL)	Position of mutation in *rpoB* gene	Position of exchanged amino acid mutation
WT strain LX-6	1390.0 ± 5.4	--	--
Rif^r strain 5r-1	1784.3 ± 7.7	A→G (1406)	Glu→Arg (469)
Rif^r strain 5r-10	1924.5 ± 7.9	A→G (1406)	Glu→Arg (469)

Rif^r strain 5r-14	847.6 ± 10.4	A→G (1445)	His→Arg (482)
Rif^r strain 5r-21	999.0 ± 6.9	A→G (1445)	His→Arg (482)
Rif^r strain 5r-24	1685.4 ± 4.1	C→T (1397)	His→Tyr (466)
Rif^r strain 5r-51	1664.6 ± 9.7	C→T (1444)	His→Tyr (482)
Rif^r strain 5r-65	1602.1 ± 8.5	C→T (1444)	His→Tyr (482)
Rif^r strain 5r-135	1627.1 ± 10.6	C→T (1444)	His→Tyr (482)

Data are expressed as mean±standard deviation (SD), n=3

Mutations conferring Rif resistance were predominantly identified in the *rpoB* gene. To further decipher these mutations, we sequenced the *rpoB* gene in eight Rif^r mutants exhibiting significant variations in neutral protease activity compared to the WT strain LX-6 (Table 2). Among these mutants, Rif^r mutant 5r-10, which displayed the highest neutral protease activity, had a Glu to Arg substitution at amino acid position 469. Conversely, Rif^r mutants 5r-14 and 5r-21 exhibited a markedly reduced neutral protease activity relative to WT LX-6, attributed to a His to Arg alteration at amino acid position 482 (Table 2).

Accordingly, the Rif^r mutant strain 5r-10, characterized by superior neutral protease production, was selected for further investigation.

To maximize neutral protease production by Rif^r mutant strain 5r-10, fermentation medium optimization was conducted by employing a single-factor approach. Through a systematic evaluation of various carbon sources, nitrogen sources and metal ions, lactose, casein and $CaCl_2$ were identified as optimal components for enhancing neutral protease production (Fig.1). The optimized fermentation medium was determined to comprise lactose (40 g/L), casein (40 g/L), $CaCl_2$ (3 g/L) and yeast extract (9.4 g/L). Under these optimized conditions, Rif^r mutant 5r-10 achieved the highest neutral protease activity of 2628.9 ± 9.7 U/mL.

(a) (b)

Unedited

(c)

Fig. 1 Effects of different carbon sources (a), nitrogen sources (b) and metal iron (c) on the protease production of *B. amyloliquefaciens* LX-6. (*) indicates statistically significant results (0.01 <*p* value < 0.05); (**) indicates highly statistically significant results (*p* value <0.01). Data are expressed as mean±SD, *n*=3.

Next, RSM was employed to improve the neutral protease activity by simultaneously optimizing the concentrations of three key nutrients in the growth medium: casein, lactose and Ca^{2+}. The central composite design involved five separate levels of each of these three factors. The actual values, coded factor levels and corresponding results are listed in Table S1. A quadratic model of the dependence of the neutral protease production yield on these three factors was built by applying multiple regression analysis to these data. In this model, the neutral protease activity (Y) could be expressed as a function of the coded values of Lactose (A), Casein (B) and Ca^{2+} (C) using the equation $Y = 2525.22-134.53 (A) - 16.72 (B) -31.01 (C) + 12.17 (AB) + 245.95 (AC) + 50.93 (BC) - 571.24 (A^2) - 210.77 (B^2) - 437.68 (C^2)$.

The adequacy of the quadratic model was assessed by using analysis of variance (ANOVA), with the results presented in Table S2. The *P* values for both linear and quadratic terms were significantly below 0.05, indicating strong correlations between neutral protease activity and the concentrations of lactose, casein and Ca^{2+}. Specifically, the interactive term AC (lactose with Ca^{2+}) was found to be significant, as opposed to AB (lactose with casein) and BC (casein with Ca^{2+}). The coefficient of determination (R^2) for the model was 0.9635, reflecting its high goodness of fit. Furthermore, the coefficient of variation (CV) was calculated at 6.54%, well below the acceptable threshold of 10%, indicating high reliability and consistency between the experimental data and the model predictions. Thus, the model demonstrated strong credibility in predicting changes in neutral protease yields based on varying concentrations of the specified nutrients.

To visualize the influences of independent variables in response values, the three-dimensional response surface for neutral protease production was plotted based on the regression model. By maintaining the third variable at its zero level, it was possible to compare the magnitude of the effects of the other two variables on the response values. A greater degree of slope curvature in the response surface indicated a stronger effect of the factors on the response value. The response surface analysis plots revealed several key insights. In the interaction plot between lactose content and casein content (Fig.S1a), the degree of curvature for lactose content was greater than that for casein content, indicating that lactose content had a more significant effect on the neutral protease yield than casein content. Similarly, in the interaction plot between lactose content and Ca^{2+} content (Fig.S1b), the curvature for lactose content was greater than that for Ca^{2+} content, suggesting a stronger influence of lactose content on the neutral protease yield compared to Ca^{2+} content. Conversely, in the interaction plot between casein content and Ca^{2+} content (Fig.S1c), the curvature for Ca^{2+} content exceeded that of casein content, indicating that Ca^{2+} content had a more substantial impact on neutral protease yield.

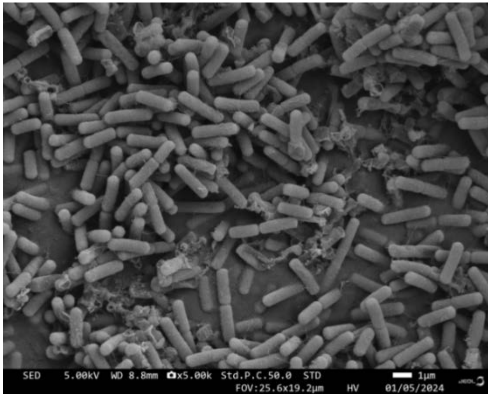
The response surface analysis predicted that the maximum production of neutral protease would be 3535.31 U/mL when the concentrations of lactose, casein and Ca^{2+} were 5.99%, 7.66%, and 0.33%, respectively. To validate this model, neutral protease production was carried out under the predicted optimal conditions. The average actual neutral protease activity of 5r-10 reached 3466.52 ± 20.2 U/mL. By optimizing the fermentation medium using RSM, the neutral protease activity of strain 5r-10 increased by 80.14% compared to the activity

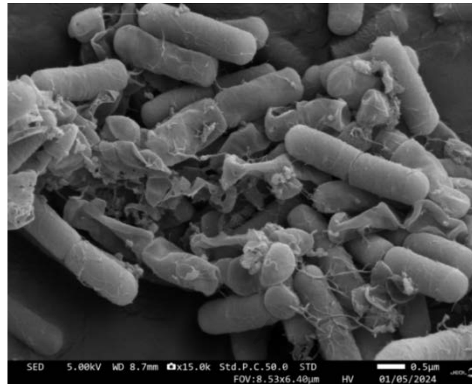
Unedited

obtained from 5r-10 in the original medium.

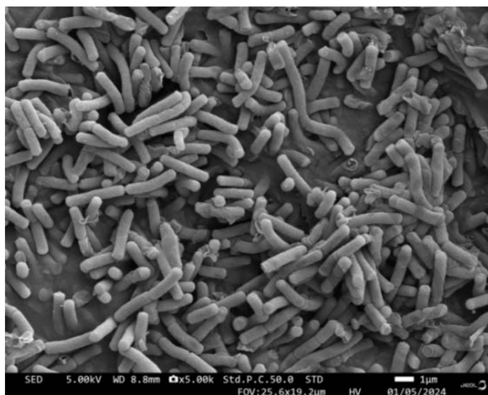
We then investigated the morphological characteristics of the WT strain *B. amyloliquefaciens* LX-6 and the mutant 5r-10 when cultured in LB broth. After two days of culture at 37°C, the mutant 5r-10 exhibited growth comparable to the WT strain LX-6. Scanning electron microscope was used to examine the morphological characteristics of strains. The WT strain LX-6 displayed visible flagella (Fig.2a), whereas the mutant 5r-10 did not form flagella and exhibited a smoother cell surface (Fig.2b).

a





b



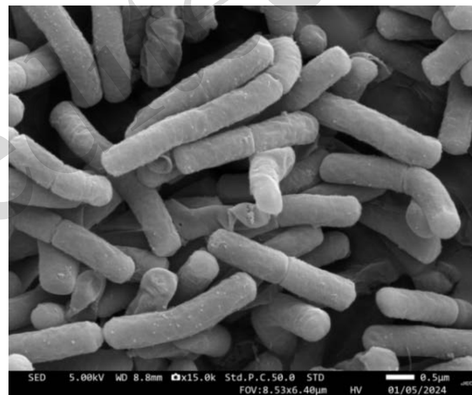


Fig. 2 Morphology of the WT strain LX-6 (a) and the Rif^r mutant strain 5r-10 (b) by using scanning electron microscope.

Both the WT strain LX-6 and the mutant 5r-10 were grown in the optimized medium and neutral protease activity was assayed every 12 hours. As shown in Fig. 3, the mutant secreted more neutral protease into the culture medium, with activity peaking at 24 hours. The highest neutral protease activity detected in the culture broth of the mutant 5r-10 was approximately 45.6% higher than that of the WT strain LX-6 (3470.67 ± 23.17 U/mL *vs*. 2383.67 ± 10.19 U/mL) ($p < 0.01$).

Fig. 3 Neutral protease activity curves of WT strain LX-6 and Rif^r mutant strain 5r-10 in the optimized medium at shake-flask level. Data are expressed as mean±SD, *n*=3.

Previous studies have suggested that SBM fermentation with *B. amyloliquefaciens* LX-6 effectively degrades ANF. To further improve the degradation rate of ANF in SBM, we investigated the effects of the WT strain LX-6 and the mutant 5r-10 on the degradation of glycinin and β-conglycinin by using SDS-PAGE, with the results shown in Fig. 4. During the initial stage of fermentation with the WT strain LX-6, some macromolecular antigen proteins began to degrade, as indicated by the weaker protein bands (33-90 kDa) of fermented SBM compared to unfermented SBM. In comparison, after 12 hours of fermentation by the mutant strain 5r-10, macromolecular proteins greater than 20 kDa were almost completely degraded into smaller molecular products. Furthermore, we assessed the degradation rates of glycinin and β-conglycinin. After 12 hours of SBM fermentation by the WT strain LX-6, the degradation rates of glycinin and β-conglycinin reached 40.2% and 28.33%, respectively. After fermentation under the same conditions by the mutant strain 5r-10, the degradation rates of glycinin and β-conglycinin were 55.33% and 37.33%, respectively. These results showed that the degradation efficiency of ANF in SBM was significantly improved after fermentation by the mutant 5r-10 (Fig.5).

Unedited

(a) (b)

Fig. 4 SDS-PAGE analyses of the macromolecular antigen proteins of SBM at different fermentation times. Lane M, standard protein molecular weight markers; lane 1, unfermented SBM; lanes 2-6: protein profiling of fermented SBM with WT strain LX-6 (a) and Rif^r mutant strain 5r-10 (b) after 12 h (lane 2), 24 h (lane 3), 36 h (lane 4), 48 h (lane 5), and 60 h (lane 6) of fermentation at 37°C.

(a) (b)

Fig. 5 Effect of SBM fermentation with WT strain LX-6 and Rif^{r} mutant strain 5r-10 on the degradation of glycinin (a) and β-conglycinin (b). Data are expressed as mean±SD, *n*=3.

Unedited

Ribosome engineering technology has been employed successfully to enhance the production of natural products by microorganisms, particularly *Streptomyces* (Li et al., 2019). However, the application of this method in *Bacillus* species remains limited. While ribosome engineering has been utilized to improve enzyme activity in *Bacillus*, most reports focused on the spectrum of mutations in spontaneous resistance mutants of *Bacillus* under specific environmental conditions (Leehan and Nicholson, 2022). To enhance the neutral protease activity of strain LX-6, we applied ribosome engineering technology to generate Rif^{r} mutants of LX-6. In conventional ribosome engineering, various antibiotics such as gentamicin, streptomycin, and erythromycin are typically used to produce and screen spontaneous resistant mutant strains; however, these methods cannot yield significant increases in neutral protease activity of the WT strain LX-6. In our study, we screened 253 spontaneous Rif^{r} mutant strains using Rif at a concentration of 5 μg/mL, and only six strains showed significantly increased enzyme activity compared to the WT strain LX-6, indicating low efficiency. Undoubtedly, it is worth considering how to further improve the efficiency of positive high-yield neutral protease of Rif^{r} mutants or how to use cumulative resistance screening strategy to further improve the neutral protease activity of strain LX-6.

The mutation sites of the *rpoB* gene in the six Rif^{r} mutant strains with significantly improved enzyme activity were located at positions H466R, E469R and H482Y. These findings are consistent with prior studies documenting that the most common amino acid changes occur in the regions from 465 to 489 in the *rpoB* gene of Rif^{r} mutants of *Bacillus* (Leehan and Nicholson, 2021). Similar mutations in the *rpoB* gene produced comparable effects on neutral protease activity; for example, in mutants 5r-1 and 5r-10 and mutants 5r-51, 5r-65 and 5r-135. Additionally, mutants 5r-14 and 5r-21, both harboring the H482R mutation, exhibited decreased neutral protease activity. Also, different mutations in the same codon of the *rpoB* gene can result in opposite effects on neutral protease activity. For instance, Rif^{r} mutants 5r-14 and 5r-21, which exhibited decreased activity, had the H482R mutation (CAC-to-CGC), while mutants 5r-51, 5r-65, and 5r-135, which exhibited higher activity than that of WT strain LX-6, had the H482Y mutation (CAC-to-TAC).

Subsequently, medium optimization for mutant 5r-10, which showed the highest increase in neutral protease activity among the six Rif^{r} mutants, was performed using the Box-Behnken design *via* RSM. The neutral protease activity of strain 5r-10 in the optimized medium was 1.49-fold of that of the WT strain LX-6 in the original medium. Interestingly, the optimized medium also benefited the WT strain LX-6, increasing its neutral protease activity from 1390±5.4 U/mL to 2383.67 ± 10.19 U/mL during the shake-flask experiments. This suggested that the introduction of the mutation into the *rpoB* gene did not significantly alter the nutritional requirements and metabolism of the strains, as both LX-6 and 5r-10 showed improved neutral protease activity in the optimized medium. Finally, the mutant 5r-10 was applied to the fermentation of SBM. Compared to fermentation with the WT strain LX-6, the degradation of macromolecular antigen proteins was accelerated, particularly for glycinin and β-conglycinin, due to the high neutral protease activity of mutant 5r-10. None-

theless, the neutral protease activity of 5r-10 was still lower than that of reported industrial strains used in SBM fermentation (Xie et al., 2022). Future efforts will focus on optimizing fermentation conditions and processes and developing a fed-batch fermentation model to further improve the neutral protease activity of mutant 5r-10. Moreover, molecular breeding strategies will be considered: we plan to clone the *npr* gene encoding neutral protease and overexpress it in the 5r-10 strain to construct an engineered strain with superior neutral protease production capabilities. To this end, follow-up studies are in progress.

Overall, the significance of this work lies in the isolation of the Rif^r mutant 5r-10 strain, which was found to exhibit higher neutral protease activity through a combination of ribosome engineering technology and medium optimization, showing a greater potential for use in SBM fermentation compared to the WT strain LX-6.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

This work was supported by Ten-thousand Talents Plan of Zhejiang Province (No. 2022R52021), Key Research and Development Program of Zhejiang Province (2021C04016), Pioneer and Leading Goose R&D Program of Zhejiang (2022C04020).

Unedited

Author contributions

Y.F.Z and X.Y.H conducted experiments. Z.M wrote the original draft and revised this article. T.H and J.T.W designed the experiments. X.P.Y polished English and checked the final version. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yifan ZHU, Xinyi HUANG, Tao HAN, Jiteng WANG, Xiaoping YU and Zheng MA declare that they have no conflict of interest.

This article does not involve any studies with human or animal subjects.

References

Cai L, Indrakumar S, Kiarie E, et al, 2015. Effects of a multi-strain *Bacillus* species-based direct-fed microbial on growth performance, nutrient digestibility, blood profile, and gut health in nursery pigs fed corn-soybean meal-based diets. J Anim Sci, 93(9):4336-4342.
https://doi.org/10.2527/jas.2015-9056

Contesini F J, Melo R R, Sato H H, 2018. An overview of *Bacillus* proteases: from production to application. Crit Rev Biotechnol, 38(3):321-334.
https://doi.org/10.1080/07388551.2017.1354354

Homayoonfal M, Malekjani N, Baeghbali V, et al, 2024. Optimization of spray drying process parameters for the food bioactive ingredients. Crit Rev Food Sci Nutr, 64(17):5631-5671.
https://doi.org/10.1080/10408398.2022.2156976

Huang X, Li H, Han T, et al, 2023. Isolation and identification of protease-producing *Bacillus amyloliquefaciens* LX-6 and its application in the solid fermentation of soybean meal. Front Bioeng Biotechnol, 11:1226988.
https://doi.org/10.3389/fbioe.2023.1226988

Koim-Puchowska B, Kłosowski G, Dróżdż-Afelt J M, et al, 2021. Influence of the medium composition and the culture conditions on surfactin biosynthesis by a native *Bacillus subtilis natto* BS19 strain. Molecules, 26(10):2985.
https://doi.org/10.3390/molecules26102985

Kumari R, Sharma N, Sharma S, et al, 2023. Production and characterization of bioactive peptides in fermented soybean meal produced using proteolytic *Bacillus* species isolated from *kinema*. Food Chem, 421:136130.
https://doi.org/10.1016/j.foodchem.2023.136130

Kurosawa K, Hosaka T, Tamehiro N, et al, 2006. Improvement of alpha-amylase production by modulation of ribosomal component protein S12 in *Bacillus subtilis* 168. Appl Environ Microbiol, 72(1):71-77.
https://doi.org/10.1128/aem.72.1.71-77.2006

Leehan J D, Nicholson W L, 2022. Environmental dependence of competitive fitness in rifampin -resistant *rpoB* mutants of *Ba-*

cillus subtilis. Appl Environ Microbiol, 88(5): e0242221.
https://doi.org/10.1128/aem.02422-21

Leehan J D, Nicholson W L, 2021. The spectrum of spontaneous rifampin resistance mutations in the *Bacillus subtilis rpoB* gene depends on the growth environment. Appl Environ Microbiol, 87(22):e0123721.
https://doi.org/10.1128/aem.01237-21

Li D, Zhang J, Tian Y, et al, 2019. Enhancement of salinomycin production by ribosome engineering in *Streptomyces albus*. Sci China Life Sci, 62(2):276-279.
https://doi.org/10.1007/s11427-018-9474-7

Nie L, Zhang R, Zhang L, et al, 2021. Mutations in the regulatory regions result in increased streptomycin resistance and keratinase synthesis in *Bacillus thuringiensis*. Arch Microbiol, 203(9):5387-5396.
https://doi.org/10.1007/s00203-021-02525-x

Peng C, Cao C, He M, , et al, 2018. Soybean glycinin and β-conglycinin-induced intestinal damage in piglets via the p38/JNK/NF-κB signaling pathway. J Agr Food Chem, 66 (36):9534-9541.
https://doi.org/10.1021/acs.jafc.8b03641

Song P, Zhang X, Wang S, et al, 2023. Microbial proteases and their applications. Front Microbiol, 14:1236368.
https://doi.org/10.3389/fmicb.2023.1236368

Suzuki H, Taketani T, Kobayashi J, et al, 2018. Antibiotic resistance mutations induced in growing cells of *Bacillus*-related thermophiles. J Antibiot (Tokyo), 71(3):382-389.
https://doi.org/10.1038/s41429-017-0003-1

Wang T, Liang C, An Y, et al, 2020. Engineering the translational machinery for biotechnology applications. Mol Biotechnol, 62(4):219-227.
https://doi.org/10.1007/s12033-020-00246-y

Xie F, Feng F, Liu D, et al, 2022. *Bacillus amyloliquefaciens* 35 M can exclusively produce and secrete proteases when cultured in soybean-meal-based medium. Colloids Surf B Biointerfaces, 209(Pt 2):112188.
https://doi.org/10.1016/j.colsurfb.2021.112188

Yao Y, Li H, Li J, et al, 2021. Anaerobic solid-state fermentation of soybean meal with *Bacillus* sp. to improve nutritional quality. Front Nutr, 8:706977.
https://doi.org/10.3389/fnut.2021.706977

Yuan L, Chang J, Yin Q, et al, 2017. Fermented soybean meal improves the growth performance, nutrient digestibility, and microbial flora in piglets. Anim Nutr, 3(1):19-24.
https://doi.org/10.1016/j.aninu.2016.11.003

Zhang J, Li H H, Chen Y F, et al, 2020. Microencapsulation of immunoglobulin Y: optimization with response surface morphology and controlled release during simulated gastrointestinal digestion. J Zhejiang Univ Sci B, 21(8):611-627.
https://doi.org/10.1631/jzus.b2000172

Zhao Y, Song Z, Ma Z, et al, 2019. Sequential improvement of rimocidin production in *Streptomyces rimosus* M527 by introduction of cumulative drug-resistance mutations. J Ind Microbiol Biotechnol, 46(5):697-708.
https://doi.org/10.1007/s10295-019-02146-w

Zhou Y, Yang X, Li Q, et al, 2023. Optimization of fermentation conditions for surfactin production by *B. subtilis* YPS-32. BMC Microbiol, 23(1):117.
https://doi.org/10.1186/s12866-023-02838-5

Zhu S, Duan Y, Huang Y, 2019. The application of ribosome engineering to natural product discovery and yield improvement in *Streptomyces*. Antibiotics (Basel), 8(3):133.
https://doi.org/10.3390/antibiotics8030133

Supplementary information

Materials and methods; Table S1-S3; Fig. S1

Unedited