Supplementary information:

Materials and methods

Materials

Polymerase chain reaction (PCR) reagents, antibiotics, gel extraction kits, and Folin-phenol reagent were purchased from TaKaRa Biotechnology Co., Ltd.

Strains and culture conditions

The strains used were as follows: the neutral protease-producing strain *B. amyloliquefaciens* LX-6, previously isolated from soil, has been deposited at the CCTCC No. M20211457.

LB medium was used for culturing *B. amyloliquefaciens* LX-6. The medium for detecting hydrolysis zones contained 50 g/L skim milk powder and 20 g/L agar powder. The original medium for neutral protease production comprised 40 g/L glucose, 40 g/L SBM, 3 g/L NaCl, and 9.4 g/L yeast extract. For the single-factor optimization experiments, different carbon sources (glycerol, starch, lactose, and sucrose), nitrogen sources (casein, ammonium sulfate, beef extract, peptone, and corn meal), and metal ions ($ZnCl_2$, $CuSO_4$, $CaCl_2$, $MgSO_4$, $MnSO_4$, and $FeSO_4$) were used to replace glucose, SBM, and NaCl, respectively, at equivalent concentrations. The optimized medium components were varied according to the Box-Behnken design, adjusting the amounts of carbon source, nitrogen source, and metal ions.

Isolation and selection of Rif^r mutants

The isolation and selection of Rif^r mutants were performed according to the similar procedure described by Zhao et al. (2019). The suspensions of *B. amyloliquefaciens* LX-6 (OD_{600}=0.5) were spread on LB medium containing different concentrations (2.5, 5, 10, and 20 μg/mL) of Rif to test the minimum inhibitory concentration (MIC) values of Rif. Concentrations of one or two times the MIC were used to select resistant mutant strains. The neutral protease activity of the isolated resistant mutant strains is an intuitive judgment based on the diameter of the hydrolysis zone on skim milk plates. Colonies with larger hydrolysis zone diameters than the WT strain LX-6 were selected for further studies. All strains were tested in three independent experiments, each performed in triplicate.

Determination of neutral protease activity

Neutral protease activity was measured according to the methods described by Huang et al. (2023).

Mapping of mutations in the *rpoB* gene of Rif^r mutants

The PCR amplification of the partial *rpoB* gene fragment (nucleotides 650-1500) was conducted by using the forward and reverse primers 5'-TTGACAGGTCAACTAGTTCA-3' and 5'-CTATTCTTTCGTTACTGCGT-3', respectively. The system and condition of PCR amplification and sequencing of PCR products were performed according to the description of Zhao et al. (2019).

Morphological observation

To evaluate morphological differentiation, *B. amyloliquefaciens* LX-6 (control) and the mutants were cultured on LB agar and incubated at 37°C. Morphological observations were made after 2 days of incubation. Microstructural observation of the WT strain and Rif^r mutant strains was performed using a scanning electron microscope (SEM) (SU-8010, China) (Huang et al., 2023).

Box-Behnken design based on response surface analysis

The Box-Behnken design using Design-Expert software was employed to evaluate the principal component factors (casein, lactose, and Ca^{2+}). The levels of the principal component factors were treated as

independent variables, with low, medium, and high experimental levels set at −1, 0, and 1 respectively. The factors and levels in the experimental protocol are shown in Table 1.

Table 1 Response surface experimental factors and levels

Factor	Level		
	−1	0	1
Lactose %	4	6	8
Casein%	4	6	8
Ca^{2+} %	0.1	0.3	0.5

SBM's fermentation by the WT strain LX-6 and its Rif^r mutants

SBM fermented by the WT strain LX-6 and its Rif^r mutants were performed according to the similar procedure described by Huang et al. (2023).

SDS-PAGE and determination of glycinin and β-conglycinin contents

SDS-PAGE manipulation and determination of glycinin and β-conglycinin contents were conducted following the standard protocol as described by Huang et al. (2023).

Data analysis

GraphPad Prism software was used to assess the significance of differences using one-way analysis of variance (ANOVA). A *P*-value of less than 0.05 was considered statistically significant.

References

Huang XY, Li HJ, 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

Zhao YF, Song ZQ, 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

Table S1 Results of Box-Behnken design

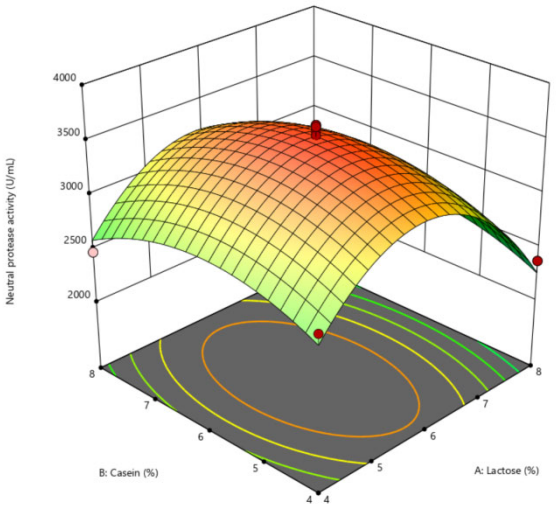
Run number	Levels of independent variables			Neutral protease activity (U/mL)
	A Lactose%	*B* Casein%	*C* Ca^{2+}%	
1	4	6	0.5	2011.94
2	4	6	0.1	2626.96
3	6	6	0.3	3288.18
4	8	6	0.5	2307.62
5	6	6	0.3	3585.82
6	6	6	0.3	3543.54
7	6	4	0.5	2350.04
8	6	4	0.1	2740.22
9	6	8	0.1	2762.34
10	4	8	0.3	2474.47
11	6	8	0.5	2657.34
12	4	4	0.3	2766.89
13	8	4	0.3	2372.44
14	6	6	0.3	3637.76
15	8	8	0.3	2148.16
16	8	6	0.1	1545.04
17	6	6	0.3	3621.24

Table S2 Analysis of variance (ANOVA) for the response surface quadratic model

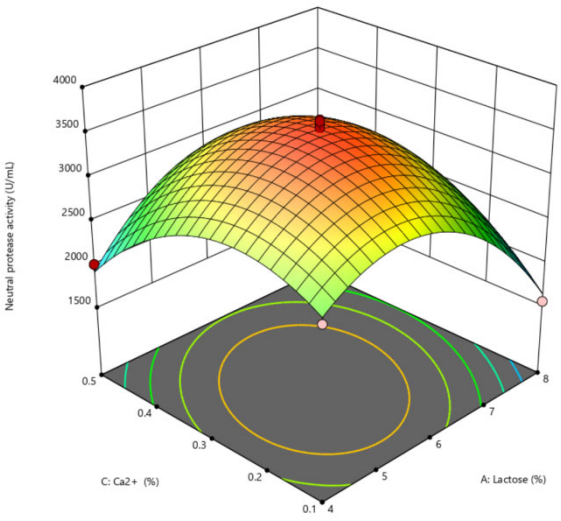
Source	Sum of squares	Degree of freedom	MS	*F*-value	*P*-value Prob.>F
Model	3.007E+06	9	3.341E+05	20.53	0.0003**
A-Lactose	1.448E+05	1	1.448E+05	8.90	0.0204*
B-Casein	2236.80	1	2236.80	0.1374	0.7218
C-Ca^{2+}	7694.20	1	7694.20	0.4728	0.5138
AB	592.19	1	592.19	0.0364	0.8541
AC	2.420E+05	1	2.420E+05	14.87	0.0062**
BC	10373.42	1	10373.42	0.6374	0.4509
A^2	1.374E+06	1	1.374E+06	84.43	<0.0001**
B^2	1.870E+05	1	1.870E+05	11.49	0.0116*
C^2	8.066E+05	1	8.066E+05	49.56	0.0002**
R^2=0.9635		R^2_{adj}=0.9166		C.V.%=6.54	

* Statistically significant at 95% of probability level; ** Statistically significant at 99% of probability level.

(a)



(b)



(c)

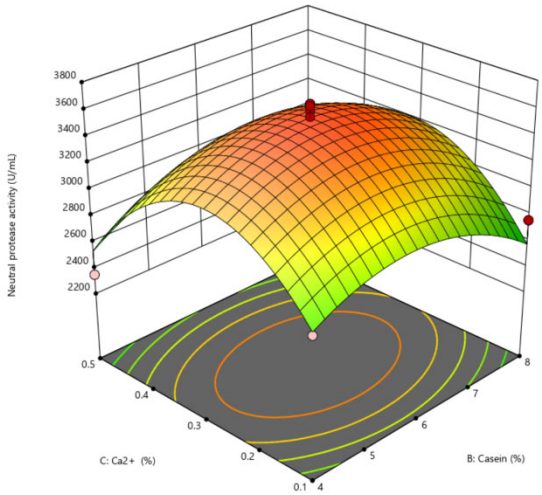


Fig. S1 Response surface plots of lactose, casein and Ca^{2+} on the neutral protease yields. Response surface plot of lactose and casein (a); response surface plot of lactose and Ca^{2+} (b); response surface plot of casein and Ca^{2+} (c).