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Full Length Article

Screening and Optimization of High-Yield Diastase-Producing Strains from Shedian Baijiu Fermented Mash

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Abstract

Diastase-producing bacteria were isolated from the fermented mash of Shedian baijiu (stilled liquors). Then the bacteria were preliminarily screened with a transparent ring method and diastatic activity was detected for secondary screening. Finally, 1 high-yield diastase-producing strain was obtained and identified as *Bacillus velezensis* by morphologic observation and 16S rDNA molecular biological identification. The enzyme-producing conditions of this strain were studied *via* single-factor experiments and optimized through response surface method. On basis of liquid fermentation medium, the optimal diastasic conditions of this strain were: soluble starch as the carbon source, peptone as the nitrogen source, initial pH 4.0, inoculation amount of 10%, and fermentation time of 3 d. The enzymatic activity after optimization was up to 120.87 ± 1.37 U/mL, or 4.06 times the initial activity.

Keywords: Shedian liquor fermented mash; High-yield diastase-producing strain; Isolated identification; Response surface methodology; Enzyme production condition optimization

Introduction

Luzhou-flavor baijiu is distilled liquor resulting from natural fermentation of grains (Long *et al.* 2018; Zou *et al.* 2018). Fermented mash is the material produced from microbiologic blending and fermentation of liquor materials (Yang *et al.* 2018a) and consists of diverse bacteria, including actinomycetales and saccharomycetes. The metabolites of these microorganisms are an important material basis accounting for the unique flavour and mouth feel of baijiu (Wang *et al.* 2008; Fang *et al.* 2019).

During the fermentation of baijiu, the microorganisms contained in fermented mash secrete various enzymes that can decompose macromolecules (*e.g.*, proteins, lipids, glucoses) in the raw materials into micromolecules (*e.g.*, amino acids, fatty acids, oligosaccharides) (Guo *et al.* 2014). In particular, diastases are an important enzymatic system in the saccharification stage of baijiu fermentation (Yu *et al.* 2015; Finley 2018). α-diastase can rapidly hydrolyze the glycosidic bonds of α-1,4 glucose and break huge starch molecules into micromolecules, fast decreasing the starch slurry viscosity and forming dextrin and minor oligosaccharides and maltoses, which are involved in the next step of fermentation (Perry *et al.* 2007). Hence, the effects of diastases on baijiu production cannot be ignored. The fermented mash is rich in diastases, so screening high-yield diastase-producing strains from the fermented mash and applying them into the liquor-making industry will largely improve the utilization rate and distillation yield of raw materials, and the functional bacteria screened out can also be applied into other fields.

In recent years, many efforts have been made to screen high-yield diastase-producing strains from baijiu Daqu (the yeast for making hard liquor) (Effront and Biodin 1916; Sun *et al.* 2019; Yao *et al.* 2019), but there is little research about screening high-yield diastase-producing strains from the fermented mash of baijiu. Only one study has focused on high-yield diastase-producing strains from the fermented mash of mild-fragrant liquors. Hence, our study is targeted at the fermented mash from Luzhou-flavor Shedian liquors and at screening high-yield diastasic strains from the fermented mash. Moreover, the enzyme-producing

conditions were investigated. The findings will theoretically underlie the culture of favorable bacteria from fermented mash.

Materials and Methods

Materials

The main material was the fermented mash collected from Shedian Laojiu Co., Ltd. (Henan Province, China).

Culture media

TruSeqTM DNA Sample Prep Kit、FastDNA SPIN Kit for Soil Kit, American MP Biomedicals Com.; AxyPrepDNA Gel Extraction Kit, American Axygen Biosciences Com.; QuantiFluor™-ST, American Promega Com.; DNA Polymerase AP221-02、Trans DNA 15KMarker, Beijing Quan shijin Biotechnology Co., Ltd.

The culture media were: a starch agar screening medium (1% peptone, 0.5% beef extract, 0.5% NaCl, 0.2% soluble starch, 2% agar powder, 121°C, sterilization for 30 min); a seed liquid medium (0.5% beef extract, 0.5% NaCl, 1% peptone, pH 7.0, 121°C, sterilization for 30 min); a liquid fermentation medium (20 g of soluble starch, 20 g of peptone, 5 g of Na_2HPO_4, 0.1 g of $MgSO_4$, 0.1 g of NaCl); an agar slant medium (10 g of peptone, 5 g of beef extract, 5 g of NaCl, 20 g of agarose, 1 L of water, pH 7.0, 121°C, sterilization for 30 min).

Instruments

The instruments included a ZQLY-300S thermostatic vibration incubator (Shanghai Zhichu Instrument Co., Ltd.), an LDZM-60KCS upright steam sterilizer (Shanghai Shenan Medical Instrument); an SW-CJ-2F double two-sided purification workbench (Suzhou Purification Equipment Co., Ltd.); an LQ-C3002 electronic balance (Shanghai Yaoxin Electronic Technology Co., Ltd.); an HH-6 thermostat water bath (Changzhou Fangke Instrument Co., Ltd.); a 101-2AS electrothermal blowing dry box (Beijing Kewei Yongxing Instrument Co., Ltd.); a DNP-9272BS-III electro-heating thermostatic cultivator (Shanghai CIMO Medical Instrument Manufacturing Ltd. Co.).

Preliminary screening

Fermented mash (10 g) was added into a triangular flask containing 90 mL of sterile water at 37°C and then oscillated at 150 r/min for 2 h. Then 1 mL of the solution was sucked using a pipette and diluted to 10^{-6}. From the diluent, 100 μL was sucked to paint a tablet, which was then placed into the thermostatic incubator for 24 h of cultivation at 37°C (Luo and Xie 2012). After that, the plate was colored with solid iodine fumigation. Specifically, iodine particles (about 1 g) were uniformly laid on a white paper, area of which should be smaller than that of a culture dish. Then the culture dish containing strains was backed-off onto the iodine particles and was then rotated for 40 s to make the coloring uniform. After the medium turned blue, the sizes of the transparent rings around the strains were observed, and the diastase-producing strains were preliminarily screened (Li *et al.* 2009). The strains with representative fungal colonies as-selected were purified on a beef extract peptone medium, and freeze-preserved in glycerole.

Afterwards, the strains were separately dripped onto the starch agar screening medium and cultured for 24 h in the thermostatic incubator, followed by solid iodine fumigation. Then the transparent rings around the diastase-producing fungal colonies were observed, and the ratio of transparent ring diameter (D) to colony diameter (d) was determined with a ruler. The strains from large D/d were selected and purified so as to preliminarily screen out the diastase-producing strains (Mao *et al.* 2015).

Secondary screening

Acquisition of rude enzyme liquids: From the slant medium, the single bacterial colonies were picked off and inoculated into the liquid seed culture medium, followed by culture for 24 h in a shaking table at 150 r/min and 37°C. After that, the seed medium was inoculated at a concentration of 10% onto a liquid fermentation medium, followed by 24 h of shaking culture at 150 r/min and 37°C (Wang *et al.* 2017a). After the fermentation liquids were centrifuged (8000 r/min, 10 min), the supernate was collected and used as the enzyme liquids for measurement of diastase activity (Liu *et al.* 2010).

Detection of enzymatic activity: Enzymatic activity was detected using a modified YOO method (Wang and Tang 1995; Shi and Jiang 1996). Specifically, 5 mL of a 0.5% (mass fraction) soluble starch solution was collected and preheated in a water bath at 40°C for 10 min; then the rude enzyme solution (0.5 mL) after appropriate dilution was added, followed by 5 min of accurate reaction in the water bath at 40°C. The reaction was terminated by adding 5 mL of 0.l mol/L H_2SO_4. Then 0.5 mL of the resulting solution was mixed with 5 mL of a sparse iodine solution for coloration, followed by measurement of absorbance at 620 nm. Also 0.5 mL of water instead of 0.5 mL of the reaction system was used as a blank, and a tube without adding the enzyme solution (the same volume of water instead) was used as a control.

Unit of enzymatic activity: 1 activity unit (U) was defined as the quantity of the enzyme needed to hydrolyze 1 mg of 0.5% starch at 40°C within 5 min.

$$\text{Enzymatic activity}\ (\text{U/mL}) = \frac{R_t - R}{R_t} \times 50 \times D$$

Where R_t is the absorbance of the control; R is he absorbance of the reaction solution; D is the dilution times of the enzyme solution and was adjusted during the

experiments to make $(R_t\text{-}R)/R_t$ fall within 0.2 and 0.7. From the tested results, the smallest absorbance corresponded to the strain with the strongest enzymatic activity.

Identification of strains

Morphologic observation: The strains screened out from the plate culture were observed in terms of colonial morphology and mobility; then single bacterial colonies after the plate culture were selected and divided by Gram's staining into Gram-positive bacteria (G^+) and Gram-negative bacteria (G^-).

Molecular biological identification

Total DNA was extracted from the strains using a Tiangen bacterial genomic DNA extraction kit and sent to polymerase chain reaction (PCR). The forward and reverse primers were the universal primers used in bacterial 16S rDNA amplification: 27F (5′-AGAGTTTGATAGAGTTTGATC-3′) /1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR conditions were: predenaturation at 94°C for 4 min; denaturation at 94°C for 1 min, annealing at 55°C for 60 s, extension at 72°C for 2 min, 30 cycles; extension at 72°C for 10 min. After that, the PCR products (5 μL) were processed by 1% agarose electrophoretic analysis to detect yield and specificity in Shanghai Sangon Biotech Co., Ltd. The sequencing results were compared, on Blast, with the data from National Center of Biotechnology Information (NCBI), and the approximate sequences were sent to phylogenetic analysis.

Optimization of enzyme-producing conditions

Single-factor assays: During single-factor analysis, the effects of carbon source (bran, corn flour, glucose, sucrose, soluble starch), nitrogen source (ammonium sulfate, urea, soybean meal powder, ammonium nitrate, peptone), initial pH (3.0, 4.0, 5.0, 6.0, 7.0), inoculation amount (4%, 6%, 8%, 10%, 12%), and fermentation time (1, 2, 3, 4, 5 d) on enzyme yields were investigated. The target strains after activation were inoculated at the amount of 10% into triangular flasks, followed by shaken culture at 150 r/min and 37°C prior to enzymatic activity measurement. Each group was repeated 3 times and the average value was used. Then the optimal enzyme-producing conditions were determined.

Response surface methodology (RSM) optimization

With diastatic activity as the response target, the experimental data were analyzed with BoxBehnken Design from RSM and with Design Expert 8.0.6. The optimal test conditions were determined and validated (Salehi *et al.* 2017).

Results

Screening of high-yield diastase-producing strains from fermented mash

The samples of Shedian Baijiu fermented mash were isolated, purified and cultured and the resulting single bacterial colonies were inoculated onto the starch agar screening medium for 1 d of culture at 37°C, followed by solid iodine fumigation. The results were illustrated in Fig. 1. After preliminary screening, over 90 diastase-producing strains were obtained. After measurement of transparent ring diameter D and strain diameter d, 7 strains with D/d > 3 were selected and sent to secondary screening (Table 1).

From the 7 strains with large transparent rings as-screened, the diastase activity of each strain was detected using the modified YOO method. Results showed the enzymatic activity of strain 5 was up to 29.78 U/mL and was larger than the other 6 strains (Table 1). Hence, strain 5 with the strongest diastase-producing ability was selected as the test strain.

Identification of high-yield diastase-producing strains from fermented mash

Morphologic observation: The strain 5 was inoculated onto the starch agar screening medium for 1 h of culture at 37°C. Then the colonial morphology was observed. It was found the strain was generally round-shaped and milk white, while its middle was bulged up and its surface was dry and folded. The results were shown in Fig. 2a. Gram's staining showed this strain was purple and Gram-positive (Fig. 2b).

Molecular biological identification: Strain 5 was analyzed *via* 16S rDNA sequencing, and the PCR products were detected by agarose gel electrophoresis (Fig. 3). The PCR fragments were in size of 1500 bp. The amplified gene sequences as-determined were compared with NCBI and a phylogenetic tree (Fig. 4) was built on Mega 6.0. Strain 5 was identified to be *Bacillus velezensis*.

Results of single-factor optimization

Effects of carbon source on enzyme yield of diastase-producing strain: The effects of carbon source on enzyme yield of the diastase-producing strain were illustrated in Fig. 5. The enzymatic activity of the strain maximized to 61.34 U/mL when the carbon source was soluble starch. Hence, the optimal carbon source for strain 5 was soluble starch.

Effects of nitrogen source on enzyme yield of strain 5: The effects of nitrogen source on enzyme yield of the diastase-producing strain were plotted in Fig. 6. The enzymatic activity of the strain maximized to 58.36 U/mL when the nitrogen source was peptone. Thus, the optimal nitrogen source for strain 5 was peptone.

Effects of initial pH on enzyme yield of strain 5: The effects of initial pH on enzyme yield of the diastase-

Table 1: Results of strain screening

No.	D/d-value	Enzymatic activity (U/mL)
5	3.33	29.78
8	3.50	13.31
9	3.78	15.23
13	3.56	13.75
19	3.03	12.95
23	3.06	12.43
2-13	3.27	12.87

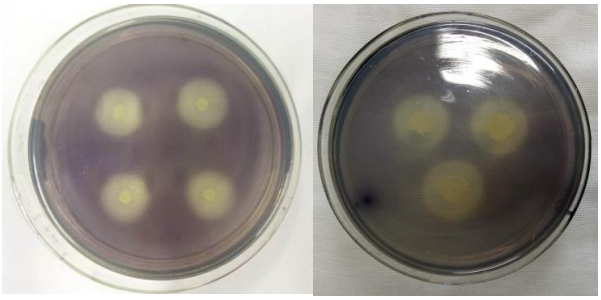

Fig. 1: Transparent rings of diastase-producing strains

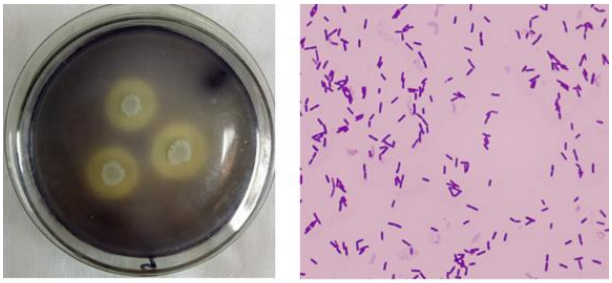

Fig. 2: Colonial morphology and Gram's staining of strain 5

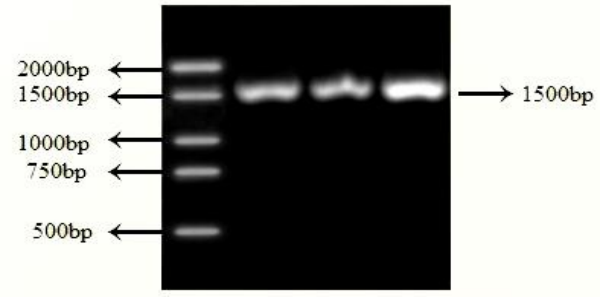

Fig. 3: PCR amplification and electrophoretic analysis

producing strain were illustrated in Fig. 7. The diastase yield of strain 5 increased with the rise of pH within 3.0 - 4.0, but then decreased with further rise at pH > 4.0. The diastase yield maximized to 73.01 U/mL at initial pH 4.0, indicating the optimal initial pH for strain 5 was 4.0.

Effects of inoculation amount on enzyme yield of strain 5: The effects of inoculation amount on enzyme yield of the diastase-producing strain were illustrated in Fig. 8. The

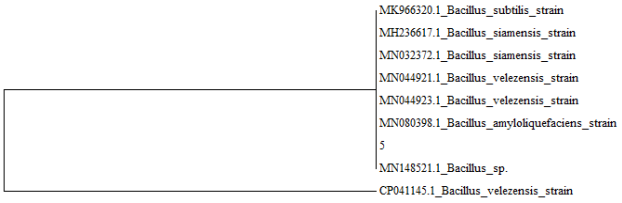

Fig. 4: Phylogenetic tree analysis

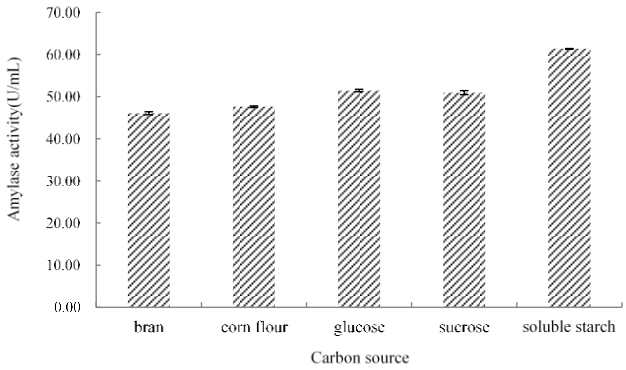

Fig. 5: Effects of carbon source on enzyme yield of strain 5

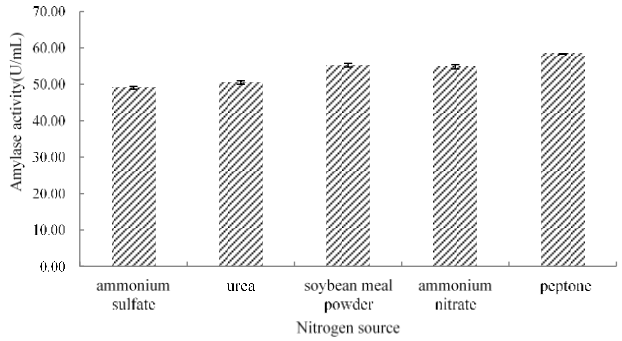

Fig. 6: Effects of nitrogen sources on enzyme yield of strain 5

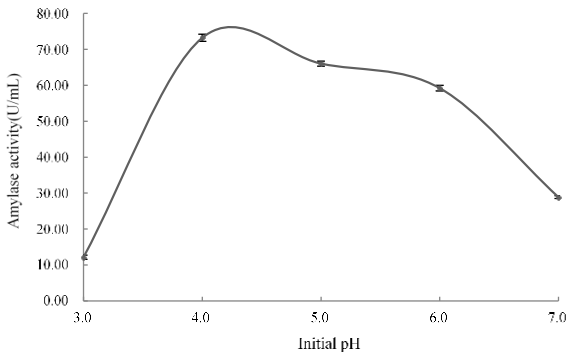

Fig. 7: Effects of initial pH on enzyme yield of strain 5

diastase yield of strain 5 increased with the rise of inoculation quantity within 4% - 10%, but then decreased with further rise of inoculation quantity at > 10%. The

diastase yield maximized to 29.01 U/mL at the inoculation amount of 10%, indicating the optimal inoculation amount for strain 5 was 10%.

Effects of fermentation time on enzyme yield of strain 5: The effects of fermentation time on enzyme yield of the diastase-producing strain were illustrated in Fig. 9. The diastase yield of strain 5 increased with the prolonging of fermentation time within 1 - 3 d, but then decreased with further rise of fermentation time at > 3 d. The diastase yield maximized to 94.37 U/mL at fermentation time of 3 d, indicating the optimal fermentation time for strain 5 was 3 d.

RSM optimization of enzyme-producing conditions

Box-Behnken design: Based on single-factor tests, we conducted Box-Behnken design on Design Expert 8.0.6. The enzyme-producing conditions of strain 5 were analyzed *via* 3-factor 3-level assays. The RSM factors and levels were listed in Table 2 and the results were shown in Table 3.

With enzymatic activity (Y) as the response value, the test results were analyzed *via* Box-Behnken Design. Then the data in Table 3 were analyzed on Design Expert 8.0.6. The regression equation of enzymatic activity with fermentation time (A), initial pH (B) and inoculation amount (C) was:

$$Y=121.23-0.70A+2.49B+1.09C+1.29AB+1.07AC+1.38BC-26.53A^2-20.37B^2-8.36C^2$$

Then the confidence and variance of the equation were analyzed (Table 4). The results of F=617.26 and $P < 0.0001$ indicate it reached the extreme significant level; the lack-of-fit as the variance was at $P=0.2427>0.05$, indicating lack-of-fit was insignificant and no lack-of-fit factor existed, which reflect the actual situations and the equation are suitable. The coefficient of determination was $R^2 =0.9956$, suggesting the test results of this equation well fitted the results of model prediction and were reliable. Hence, the results are reliable, and this equation can be used to analyze and predict the enzymatic activity of this strain.

RSM and contour analysis of enzymatic activity: The RSM curves and contour curves showing the between-two interactive effects of fermentation time, initial pH and inoculation amount on enzymatic activity were plotted in Fig. 10–12.

Determination of optimal fermentation conditions and verification

The effects of B, A^2, B^2 and C^2 were all extremely significant ($P < 0.01$) and the effects ranked as B>C>A, or namely initial pH > inoculation amount > fermentation time (Table 4). The optimal culture conditions were found to be: fermentation time of 2.99 d, initial pH at 4.06, and inoculation amount of 10.14%. The enzymatic activity under the optimal conditions was predicted to be 121.35 U/mL. To validate the effectiveness of RSM and considering the convenience of actual operations, we modified these factors to be fermentation time of 3 d, initial pH at 4 and inoculation amount of 10%. Then 3 parallel tests were conducted under these conditions, and the actual enzymatic activity was 120.87 ± 1.37 U/mL, which was basically close to the predicted value.

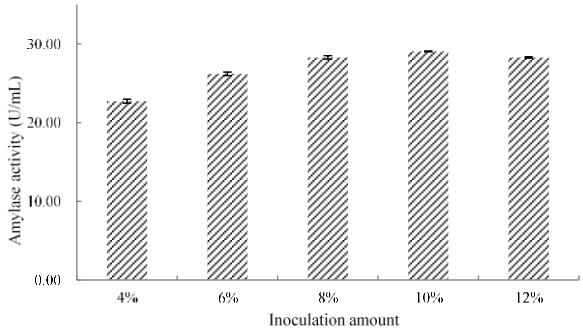

Fig. 8: Effects of inoculation amount on enzyme yield of strain 5

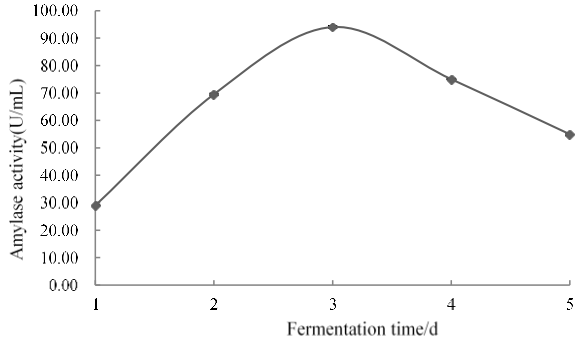

Fig. 9: Effects of fermentation time on enzyme yield of strain 5

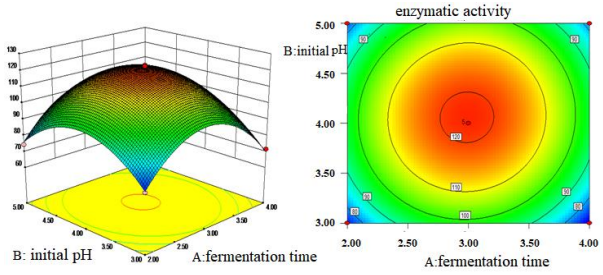

Fig. 10: The RSM curves and contour curves showing the interactive effects of fermentation time and initial pH on enzymatic activity

Discussion

Many efforts have been made to screen high-yield diastase-producing strains from baijiu Daqu or oceans, but little research has focused on the fermented mash of baijiu. The diastase-generating yeast screened out from the fermented

Table 2: RSM factors and levels

Level	Factor		
	A-Fermentation time/d	B-Initial pH	C-Inoculation amount/%
-1	2	3	8
0	3	4	10
1	4	5	12

Table 3: Response surface test design and results

No.	A-Fermentation time/d	B-Initial pH	C-Inoculation amount/%	Enzymatic activity U/mL
1	4	3	10	71.6
2	3	4	10	120.45
3	4	5	10	78.23
4	3	4	10	119.41
5	3	3	8	89.9
6	4	4	12	86.58
7	2	4	8	88.24
8	2	3	10	73.01
9	3	5	8	93.05
10	3	4	10	122.5
11	2	5	10	74.48
12	4	4	8	82.11
13	3	4	10	123.14
14	2	4	12	88.41
15	3	5	12	97.85
16	3	4	10	120.65
17	3	3	12	89.2

Table 4: Analysis of variance of regression equation

Sources	SS	DF	MS	F-value	P-value	S	
Model	5555.37	9	617.26	617.26	176.17	< 0.0001	Extremely significant
A	3.95	1	3.95	3.95	1.13	0.3237	
B	49.50	1	49.50	49.50	14.13	0.0071	Extremely significant
C	9.55	1	9.55	9.55	2.73	0.1428	
AB	6.66	1	6.66	6.66	1.90	0.2105	
AC	4.62	1	4.62	4.62	1.32	0.2885	
BC	7.56	1	7.56	7.56	2.16	0.1853	
A^2	2964.10	1	2964.10	2964.10	845.95	< 0.0001	Extremely significant
B^2	1746.67	1	1746.67	1746.67	498.50	< 0.0001	Extremely significant
C^2	294.45	1	294.45	294.45	84.03	< 0.0001	Extremely significant
Residual	24.53	7	3.50	3.50			
Lack of fit	15.01	3	5.00	5.00	2.10	0.2427	Not significant
Pure error	9.52	4	2.38	2.38			
Total	5579.90	16					

mash of mild fragrant baijiu had the enzymatic activity up to 118 U/mL (Yu *et al.* 2015). The α-diastase-producing bacterium isolated from oceans had the enzymatic activity of 77.44 U/mL (Wang *et al.* 2017b). The diastase-producing bacterium isolated from lakes had the enzymatic activity of 147.53 U/mL (Henipigul *et al.* 2017). Thus, the high-yield diastase-producing strain screened out in this study is of high ability. If this strain can be further mutagenized or gene-recombined, its enzymatic activity will be improved.

At present, a number of bacilli have been applied into agriculture, industry and environmental protection and have made great contribution to humans and the society (Guo *et al.* 2007; Pérez-García *et al.* 2011; Panda *et al.* 2014; Sumi *et al.* 2015). Bacilli also play very important roles in the field of baijiu making (Luo *et al.* 2019). For instance, the bacilli identified in fermented mash can produce multiple enzymes, including diastase, protease and cellulose (Gashaw and Gessesse 1997; Wang *et al.* 2009). In particular, diastases are a critical part and appropriate control of diastase-producing bacilli during baijiu fermentation can improve the utilization rate and distillation yield of starch raw materials. As reported, bacilli can produce higher alcohols, higher ketones and other fragrant compounds, which are at trace levels in baijiu and play the roles of fragrance appending, assisting and seasoning in Luzhou-flavor liquors (Yang *et al.* 2018b). As reported, the fragrance of Luzhou-flavor liquors mainly originates from the esterified fragrance-enhancing phase at late fermentation, and bacilli bloom at this phase and become the predominant bacterial colonies of fermented mash, thus playing an important role in fragrant enhancement (Hu *et al.* 2014). Thus, in addition to the enzyme-producing ability, bacilli can provide baijiu with intense fragrance. The bacillus screened out in our study has broad application prospects in baijiu production.

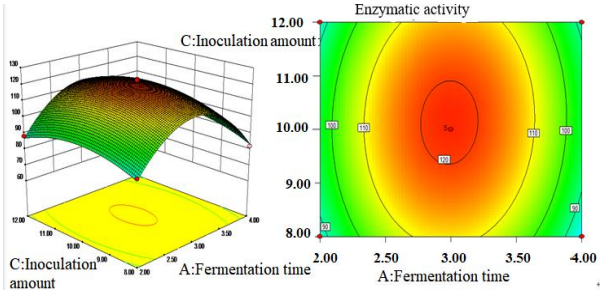

Fig. 11: The RSM curves and contour curves showing the interactive effects of fermentation time and inoculation amount on enzymatic activity.

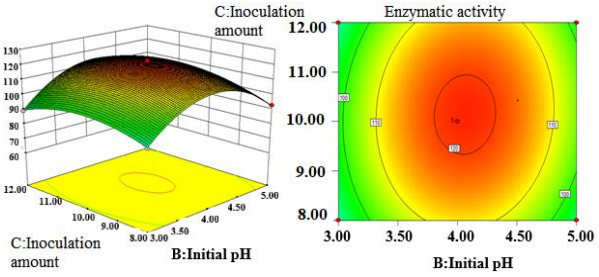

Fig. 12: The RSM curves and contour curves showing the interactive effects of inoculation amount and initial pH on enzymatic activity

Conclusion

The strains were screened preliminarily with the transparent ring method and secondly with diastatic activity measurement. Finally, 1 high-yield diastase-producing strain was screened out from Shedian baijiu fermented mash and its initial enzymatic activity was 29.78 U/mL. Morphologic observation and 16S rDNA molecular biological analysis identified it as *Bacillus velezensis*. The enzyme-producing conditions were then studied through single-factor tests and optimized by RSM to be: soluble starch as carbon source, peptone as nitrogen source, initial pH at 4, inoculation amount of 10%, and fermentation time of 3 d. Under these conditions, the enzymatic activity was up to 120.87 ± 1.37 U/mL, which increased by 4.06 times from the initial level, indicating this strain has potential industrial application values.

Acknowledgments

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Author Contributions

Yanbo Liu, Zhijun Zhao and Chunmei Pan planned the experiments, Wenjuan Zhang, Wenning Gu and Xian Wang interpreted the results, Yanbo Liu and Chunmei Pan made the write up and Xiyu Sun statistically analyzed the data and made illustrations

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