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Cloning, expression and medium optimization of validamycin glycosyltransferase from *Streptomyces hygroscopicus* var. *jinggangensis* for the biotransformation of validoxylamine A to produce validamycin A using free resting cells

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HIGHLIGHTS

- Cloning and expression of *valG* from *Streptomyces hygroscopicus* var. *jinggangensis*.
- Biotransformation of validoxylamine A to validamycin A with cellobiose as the sugar donor.
- Using resting cells instead of validamycin glycosyltransferase.
- Improving the content of validamycin A in the products.
- Effective optimization of the culture medium with response surface methodology.

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ABSTRACT

Validamycin A is widely used to control *Basidiomycetes*, which causes sheath blight disease in rice, potatoes, vegetables, and other crops as well as dumping-off disease in vegetable seedlings, cotton, sugar beets, and other plants. In order to improve the content of validamycin A in the commercial products, *valG* from *Streptomyces hygroscopicus* was successfully cloned into *Escherichia coli* BL21(DE3) and was directly employed as the biocatalyst in the biotransformation from validoxylamine A to validamycin A with the existence of D-cellobiose using the free resting cells in the present study. The fermentation medium was optimized through single factor experiment and response surface method. With the optimized medium, which contained lactose 4.7 g/L, yeast extract 49.5 g/L, ammonium chloride 2.7 g/L, potassium phosphate buffer solution 110 mL/L, Ca^{2+} 0.0352 g/L, the biomass yield and enzyme activity reached 5.5 g/L and 1.49 U/mL, respectively, which were nearly twice higher than those with initial medium.

1. Introduction

Validamycin A, the main component of the validamycin complex, the family of weakly basic C_7N-aminocyclitol-containing antibiotics, was first isolated from *Streptomyces hygroscopicus* var. *limoneus* (Iwasa et al., 1970, 1971; Horii et al., 1972), and later from *S. hygroscopicus* var. *jinggangensis* 5008 (Liao et al., 2009; Wei et al., 2010; Xu et al., 2008; Xu et al., 2009a, b; Yu et al., 2005). It is widely used to control *Basidiomycetes*, especially *Pellicularia sasakii* (*Rhizoctonia solani*) which causes sheath blight disease in rice, potatoes, vegetables, and other crops as well as dumping-off disease in vegetable seedlings, cotton, sugar beets, rice, and other plants (Nioh and Mizushima, 1974; Robson et al., 1998). Its potent antifungal activity is attributed to validoxylamine A. Validoxylamine A is a strong inhibitor of trehalase due to its structural similarity to trehalose, the primary storage carbohydrate in many fungi (Iwasa et al., 1970). It is more active than validamycin A against fungi *in vitro* tests, but *in vivo* tests suggested the opposite (Kameda et al., 1987). It is proposed that the glucose moiety on validamycin A is required for the efficiency entry into fungal hyphae, in which it is hydrolyzed to produce the more active form, validoxylamine A (Asano et al., 1991). Validamycin A was also used as the source of valienamine, a pharmaceutically important precursor for the production of the antidiabetic drug, voglibose (Basen), whose mechanism of action is to another antidiabetic agent, acarbose (Chen et al., 2003; Chen et al., 2006).

Therefore, validamycins were produced in large scale in China with many product specifications, such as products of 40% (W/W) validamycin A and 60% (W/W) validamycin A. However, it is

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hard to improve the content of validamycin A more than 70% because of the purifying cost. In validamycin mixture, there are about 60% validamycin A, 20–30% validoxylamine A, 5–8% other members of validamycins, such as validamycin B–H, and 2–3% other members of validoxylamines, such as B, G. Zhou et al. (2011) reported that 3 g/L validamycin A and 0.5 g/L validoxylamine A accumulated at the end of the fermentation during the fermentation of wild-type *S. hygroscopicus* var. *jinggangensis* 5008. The similar phenomenon was also observed in the high-yielding validamycin A producer TL01, with 18 g/L validamycin A and 4 g/L validoxylamine A produced. Furthermore, the molar ratio of validoxylamine A/validamycin A was even higher in the high-yielding strains than that in low-yielding strains. Although the validamycin A content could be increased by adding UDP-glucose to decrease the validoxylamine A accumulation (Zhou et al., 2011), the cost of UDP-glucose is too high to be used in the industrial scale. The other way to solve the problem is to biotransform the validoxylamine A to validamycin A using validamycin glycosyltransferase.

Glycosyltransferases catalyze the transfer of a sugar moiety from a nucleotidyldiphosphate (NDP)-sugar to an acceptor, which could be a growing oligosaccharide, a lipid, a protein, or a small molecule. On the basis of tertiary structure analysis, glycosyltransferases have been divided into two superfamilies, known as glycosyltransferase-A and glycosyltransferase-B (Bourne and Henrissat, 2001). Members of the glycosyltransferase-A superfamily contain two dissimilar domains, one involved in the recognition of the NDP-sugar and the other in the recognition of the acceptor molecule. Most of the glycosyltransferases in the Leloir pathway that reside in the Golgi apparatus and the endoplasmic reticulum belong to this family. The glycosyltransferase-B superfamily is remarkably diverse and contains members that are extremely promiscuous to their NDP-sugar donors (Barton et al., 2002; Jiang et al., 2001). The glycosyltransferase-B family consists mostly of prokaryotic enzymes which glycosylate secondary metabolites to produce active natural products, as well as some glycosyltransferases from the primary pathways. For *S. hygroscopicus* subsp. *jinggangensis* 5008, the complete biosynthetic gene clusters have been identified (Bai et al., 2006). Among the proteins believed to be directly involved in the biosynthesis, validamycin glycosyltransferase has been characterized both *in vivo* and *in vitro* as a glycosyltransferase that catalyzes the conversion of validoxylamine A to validamycin A using UDP-glucose as the sugar donor (Bai et al., 2006). Interestingly, validamycin glycosyltransferase belongs to the glycosyltransferase-A family of glycosyltransferases, even though it functions in secondary metabolism. Later, *val*G was cloned from *S. hygroscopicus* subsp. *jinggangensis* 5008 and expressed to produce validamycin glycosyltransferase (Xu et al., 2008). And the validamycin glycosyltransferase was applied in biotransformation of validoxylamine A to validamycin A or 4''-*epi*-validamycin A with UDP-glucose as the sugar donor.

However, in the biotransformation process, the enzyme was used as the catalyst and the UDP-glucose as the sugar donor. It is not suitable to be applied in the industrial production of validamycin A because of the production cost, including the purification of the enzyme and purchase of UDP-glucose. If the resting cells could be used directly instead of the enzyme, the cost would be decreased definitely. Another sugar donor, D-cellobiose was reported to be used in β-glucosidation of validoxylamine A (Kameda et al., 1980). If D-cellobiose could be utilized as the sugar donor instead of UDP-glucose, the cost of the validamycin A production would be decreased again because D-cellobiose is much cheaper than UDP-glucose.

Our present study was focused on cloning and expression of validamycin glycosyltransferase in *Escherichia coli* and its application in the biotransformation of validoxylamine A to validamycin A with D-cellobiose as the sugar donor using the resting cells. The conditions for the expression of validamycin glycosyltransferase were optimized to improve the effectiveness of biocatalysis.

2. Methods

2.1. Strains and plasmids

The validamycin producing strain, *S. hygroscopicus* subsp. *jinggangensis* was maintained in our laboratory. *E. coli* BL21 (DE3), *E. coli* DH5α, pET-28b (+) were purchased from Novagen (Philadelphia, PA).

2.2. Chemicals

Restriction enzymes, T4 DNA ligase, Taq DNA polymerase were purchased from TaKaRa Bio. Inc.. Kanamycin was purchased from Sigma. Validamycin A and validoxylamine A were given by Zhejiang Tonglu Huifeng Biochemistry Co., Ltd. as a gift. All other chemicals were purchased in the local market and analytically pure.

2.3. Media and culture conditions

S. hygroscopicus was grown in Gause's Synthetic agar at 28 °C for activation; for mycelium preparation, *S. hygroscopicus* was grown in Bennett's medium (glucose 10 g/L, N-Z amino type A 2 g/L, yeast extract 1 g/L, Beef extract, 1 g/L, pH 7.3) at 28 °C; *E coli* BL21(DE3) and *E coli* DH5α were cultured in Luria Broth (LB) at 37 °C in the presence of kanamycin (50 μg/mL); The media, LB, 2 × YT, TB, M9, SOB, and SOC, were prepared according to the literature (Sambrook, 2001).

2.4. Construction of pET28b-valG

The total DNA of *S. hygroscopicus* subsp. *Jinggangensis* was taken as the template, a pair of primers was designed according to the base sequence (Accession number: ABA41511) of glycosyltransferase as follows, 5′-GGAATTCCATATGATGCCCGGTGCG ACATC-3′, 5′-GGAATTCTCAGTCACCGCGAAGAGAC-3′ (the underlined part were endonuclease digestion sites). PCR amplification was under the following condition: initial denaturation at 94 °C for 2 min; denaturation at 94 °C for 45 s; annealing at 61.4 °C for 30 s; extension at 72 °C for 1 min for 30 cycles; extension at 72 °C for 10 min.

PCR-amplified fragments were digested with *Nde*I and *Eco*RI, and ligated into *Nde*I/*Eco*RI-digested pET28b to construct an expression plasmid pET28b-*val*G. Plasmid pET28b-valG was then transformed to *E. coli* DH5α, *E. coli* BL21 (DE3) to obtain BL21 (DE3)-pET28b-*val*G.

BL21 (DE3)-pET28b-*val*G was grown at 37 °C in LB medium containing kanamycin with a concentration of 50 μg/mL. When OD_{600} reached 0.6, lactose with a final concentration of 100 mM was added, and then the bacteria were cultivated for another 10 h. Cells were harvested by centrifuging at 10,000 rpm for 5 min, then were washed with pure water twice and stored at 4 °C for the further use.

2.5. Analysis methods

The enzyme activity was assayed as follows. 0.1 g cells were added into EP tube with 1 mL reaction buffer containing 0.1% validoxylamine A, 1% D-cellobiose, and 0.1 M PBS (pH 6.0) buffer (Kameda et al., 1980). After 24 h at 37 °C, the reaction mixture was centrifuged at 12,000 rpm for 5 min. Then a HPLC method was employed to detect the yield of validamycin A in the supernatant. One unit of validamycin glycosyltransferase activity was

a

a. Result of the amplified fragment with electrophoresis. Lane 1 and lane 2: the cloned DNA fragment; Lane 3: DNA marker.

b

5'-ATGCCCGGTGCGACATCCCATGTGCCACTGCTCTCGGTCGTCATCCCGACATACA
ACCGAGCCGCCCTCCTCGACCGGACGTTAGGCACGCTGGCCCGTCAGACGACGGC
GCTCGAGGACTTCGAGGTCGTGGTGTCCGACGATGGCTCGACAGACACCACCCGC
GACGTAGTGCGGTCCTACGAGGACCGGCTGCGCATCAAGTACGTCTTCCAGGAAG
ACCTTGGATACCGAGTCGCCAGCGCGCGCAACGGTGGGGCACGCCTGGCCTCCGC
GCCACTCCTGGCCTTCCTCGACACTGGTGTCCTGGCGGGGCCGCAGTACGTCCAG
TCGGTCCTTGCCGCCCATGCCGGGCCGGCGCCCGCCAAGGTGGTCCTCGGCTGCT
GCTACGGCTACGATCCGCGGAATCCCCATCCCGAACTCCACTCCCTTGTCGAGGAG
TTCCCCCCCGGAGGAGGCCGTGAGAAGGGTCGGGGACGCCCCCTGGTTCCAGGACA
TGCGGCTTCCGGAGTTCACTGCGGTCGACTTCGACCTAAGCCGTATGCACATGCCC
TGGCTGTGGTTCTGGACGCTCAACGTGTCGCTGCCGGCAGCCGACTTCTGGCGGG
TCGGCGGGTTCGACGAGGACTTCACCGGCTGGGGCGGGGAGGACATCGAACTCG
GCTACCGGCTGCACGCACACGGCATCCCCATGACGGTCTCACGTGAGAGCTGGGG
GATCGAAGCGCCGCACGAGCGCACTCATGAGGCGAACGTCTCCTCCCTCATGCTC
AATTGCGACCGGTTCGTCCGCAAGCACCCCTCCCTGCTCCCCGAGCTGTTCTGGGC
GGTGACCAACAGGGGCATCTTCGGCTCCGTCGAGACTGAGCGGCTTCGCTTCGAG
GAATGGGCAAGCCAGGCCCGCGGGCAACAGGTCCTGGACGAGATCGCCATCGGGT
TGGACACCCTGCCCCCGTCGCAGCACACCCAGCGGGTGGCGGTGTTCGGAAGCGG
CACGGAGGGGCTTCCCATCACTCCGCGGCAGAATGTCGAACTCTTCCTGTGTGATT
ACGATGAGGGGGTGCTCGCACGGCAAGAGAGCCGCGACGATGCCGCCGTCTCGA
CCTGGCATCTGTCCGGGCTGCGTACCCCCTGGCCCGACCAGCACTTCGACCTGGTC
ATCATCACCTCGCGCATGGACGGGCCCCGGCAGGCATGGGGCGAGGCGTTCACCA
AGGAGGCGCACCGGATCGCGAGCTCAGTCGTCGAGCCGTCTCTTCGCGGTGACTG
A-3’

b. Sequence result of the amplified fragment.

Fig. 1. Results: Cloning of *valG* from *S. hygroscopicus* subsp. *Jinggangensis*.

defined as the amount of enzyme that produced 1 μmol of validoxylamine A equivalent per minute.

HPLC analysis for validamycin A and validoxylamine A was performed on LC-20AT system (Shimadzu) combined with SPD-20A UV detector (Shimadzu); the conditions were: column of Thermo ODS-2 HYPERSIL (C18) column (4.6 mm × 250 mm, particle size 5 μm), mobile phase of MeOH-Na_2HPO_4 buffer (pH 7.0) (3:97, v/v), flow rate of 1.0 mL min^{-1}, and wavelength of 210 nm.

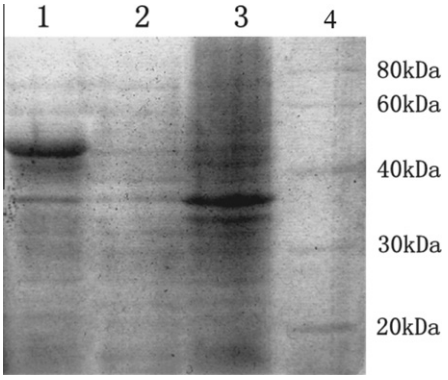

Fig. 2. Effect of the optimal inducing conditions of lactose on protein expression. Lane 1: Recombinant strain induced by lactose for 10h; Lane 2: Non-induced recombinant strain; Lane 3: BL21 only containing pET-28b; Lane 4: Low molecular weight standards.

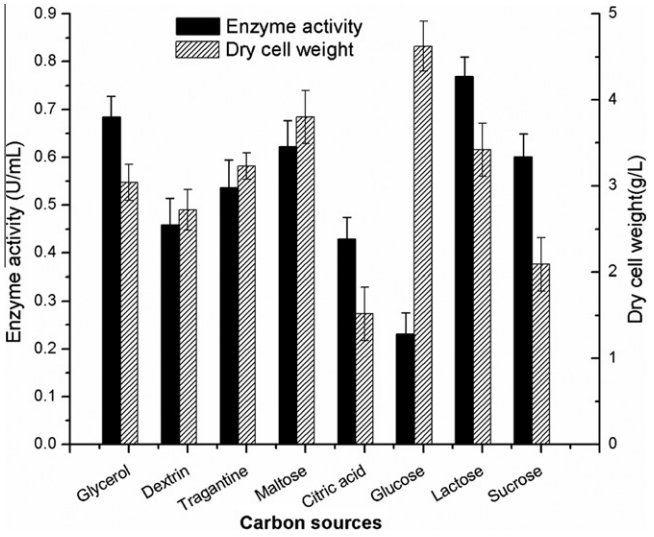

Fig. 3. Effect of carbon sources on biomass yield and enzyme activity.

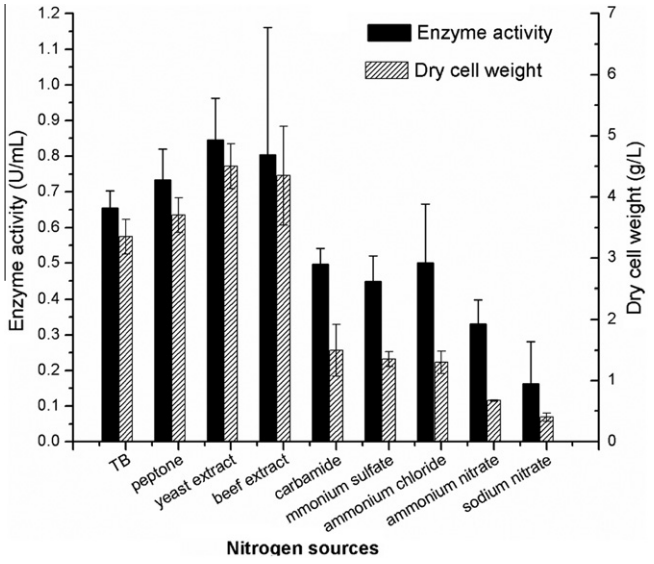

Fig. 4. Effect of single nitrogen source on biomass yield and enzyme activity.

Table 1
Effects of composite nitrogen sources on the biomass yield and enzyme activity.

Nitrogen sources	Concentration (g/L)	Enzyme activity (U/mL)	Error line	Biomass yield (g/L)	Error line
Yeast extract	42.7	0.97	0.28	3.82	0.62
Yeast extract, peptone	16.7, 16.7	0.94	0.31	4.16	0.22
Yeast extract, beef extract	17.5, 17.5	0.49	0.09	2.47	0.39
Yeast extract, beef extract	25.0, 12.0	0.51	0.11	3.20	0.11
Yeast extract, ammonium sulfate	38.0, 2.0	1.03	0.13	4.30	0.13
Yeast extract, ammonium chloride	37.0, 2.0	1.08	0.15	4.93	0.23
Yeast extract, peptone, ammonium chloride	14.0, 14.0, 2.0	0.73	0.09	3.79	0.22
Yeast extract, beef extract, ammonium chloride	15.0, 15.0, 2.0	0.62	0.28	2.59	0.29

Table 2
Effect of adding volume of nitrogen source on enzyme production and biomass yield.

Concentration of nitrogen	Enzyme activity (U/mL)	Biomass yield (g/L)
18.5 g/L yeast extract, 1.0 g/L ammonium chloride	1.020	3.75
27.7 g/L yeast extract, 1.5 g/L ammonium chloride	1.032	4.46
37.0 g/L yeast extract, 2.0 g/L ammonium chloride	1.051	4.61
46.2 g/L yeast extract, 2.5 g/L ammonium chloride	1.264	4.95
55.4 g/L yeast extract, 3.0 g/L ammonium chloride	1.124	4.74

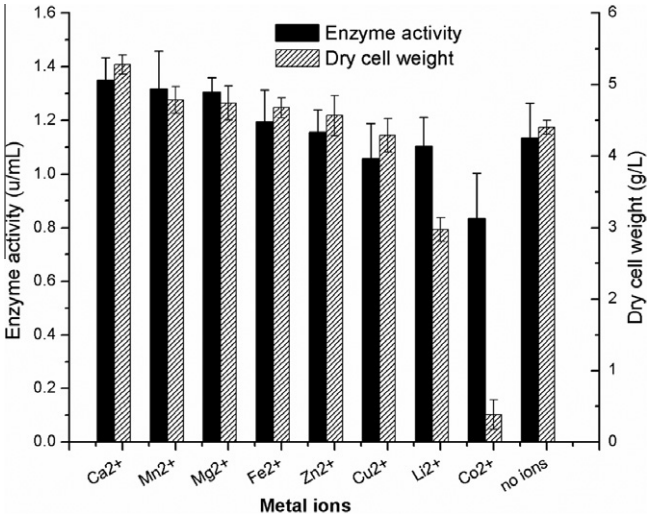

Fig. 5. Effect of ions on biomass yield and enzyme activity.

Biomass was assayed by weighing the dry cell weight. The broth was first centrifuged, then poured out the supernatant and washed with pure water for 3 times. The biomass was dried in the oven at 105 °C for 4 h. Finally, the biomass was weighed as the dry cell weight.

Table 3
Box–Behnken design of 4 factors and 3 levels and results.

Running order	Lactoseg (L)		Yeast extract/ammonium chloride (g/L)		Potassium phosphate buffer solution (mL/L)		Ca^{2+} (mM)		Enzyme activity (U/mL)
	Code X_1	X_1	Code X_2	X_2	Code X_3	X_3	Code X_4	X_4	
1	0	5	0	46.2 and 2.5	−1	50	1	2	1.372
2	0	5	0	46.2 and 2.5	−1	50	−1	0	1.342
3	1	7.5	0	46.2 and 2.5	1	150	0	1	1.291
4	0	5	1	55.4 and 3.0	1	150	0	1	1.445
5	−1	2.5	0	46.2 and 2.5	0	100	1	2	1.330
6	0	5	−1	37.0 and 2.0	1	150	0	1	1.300
7	1	7.5	−1	37.0 and 2.0	0	100	0	1	1.209
8	0	5	1	55.4 and 3.0	0	100	−1	0	1.456
9	0	5	0	46.2 and 2.5	0	100	0	1	1.502
10	0	5	0	46.2 and 2.5	1	150	1	2	1.319
11	−1	2.5	0	46.2 and 2.5	1	150	0	1	1.395
12	1	7.5	0	46.2 and 2.5	−1	50	0	1	1.321
13	0	5	−1	37.0 and 2.0	0	100	−1	0	1.359
14	0	5	0	46.2 and 2.5	0	100	0	1	1.510
15	−1	2.5	1	55.4 and 3.0	0	100	0	1	1.350
16	−1	2.5	−1	37.0 and 2.0	0	100	0	1	1.305
17	1	7.5	0	46.2 and 2.5	0	100	1	2	1.261
18	0	5	1	55.4 and 3.0	0	100	1	2	1.412
19	0	5	1	55.4 and 3.0	−1	50	0	1	1.468
20	1	7.5	0	46.2 and 2.5	0	100	-1	0	1.282
21	0	5	0	46.2 and 2.5	0	100	0	1	1.507
22	0	5	−1	37.0 and 2.0	−1	50	0	1	1.320
23	0	5	0	46.2 and 2.5	1	150	-1	0	1.441
24	−1	2.5	0	46.2 and 2.5	−1	50	0	1	1.400
25	0	5	−1	37.0 and 2.0	0	100	1	2	1.304
26	1	7.5	1	55.4 and 3.0	0	100	0	1	1.310
27	−1	2.5	0	46.2 and 2.5	0	100	−1	0	1.342

SDS–PAGE was performed in a 12.5% (w/v) polyacrylamide gel. The marker proteins were protein molecular weight standards in the range of 14.4–97 kDa (GE Healthcare). Protein bands were visualized by Coomassie brilliant blue R-250 staining.

2.6. Optimization of composition of fermentation medium used for *valG*

The composition of fermentation medium, including carbon sources, nitrogen sources and inorganic salts on dry cell weight and validamycin glycosyltransferase activity were tested and identified via the response surface design experiment using 250-mL shaking flasks.

3. Results and discussion

3.1. Cloning and sequence analysis

According to the literature (Bai et al., 2006), the sequence length of validamycin glycosyltransferase *S. hygroscopicus* subsp. *Jinggangensis* was 1269 bp and 422 amino acid residues were encoded by this gene. An about 1200 bp DNA fragment band was amplified as shown in Fig. 1a. The sequence result of the amplified fragment was shown in Fig. 1b. The sequence, 1269 bp, was the same as that reported in the literature (Bai et al., 2006). Therefore, the obtained *val*G was used as the starting material for the further experiments.

3.2. Overexpression of valG in E. coli

The whole cell SDS–PAGE analysis was carried out after inducing expression. Fig. 2 indicated that there was a clear expressing protein of 47 kDa and there was no sign of expression in the control and non-induced group.

Table 4
Box–Behnken experimental design variance analysis results.

Source	Degrees of freedom	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	14	0.155622	0.155622	0.011116	12.71	0.000
Linear	4	0.055553	0.055553	0.013888	15.88	0.000
X_1	1	0.016725	0.016725	0.016725	19.12	0.001
X_2	1	0.034561	0.034561	0.034561	39.52	0.000
X_3	1	0.000085	0.000085	0.000085	0.10	0.760
X_4	1	0.004181	0.004181	0.004181	4.78	0.049
Square	4	0.093299	0.093299	0.023325	26.67	0.000
X_1X_1	1	0.051003	0.084560	0.084560	96.69	0.000
X_2X_2	1	0.010379	0.026633	0.026633	30.45	0.000
X_3X_3	1	0.003561	0.012632	0.012632	14.44	0.003
X_4X_4	1	0.028356	0.028356	0.028356	32.42	0.000
Interaction	6	0.006769	0.006769	0.001128	1.29	0.332
X_1X_2	1	0.000784	0.000784	0.000784	0.90	0.362
X_1X_3	1	0.000156	0.000156	0.000156	0.18	0.680
X_1X_4	1	0.000020	0.000020	0.000020	0.02	0.882
X_2X_3	1	0.000002	0.000002	0.000002	0.00	0.960
X_2X_4	1	0.000030	0.000030	0.000030	0.03	0.856
X_3X_4	1	0.005776	0.005776	0.005776	6.60	0.025
Residual error	12	0.010494	0.010494	0.000875		
Lack of fit	10	0.010462	0.010462	0.001046	64.05	0.015
Pure error	2	0.000033	0.000033	0.000016		
Cor. total	26	0.166116				

3.3. Effect of basic medium on biomass yield

In order to determine the optimal medium for cells to grow and produce enzyme, the effect of liquid medium of LB, 2 × YT, TB, M9, SOB and SOC on growth of recombinant strain BL21(DE3)-pET28-*valG* were investigated. The results indicated that TB medium was the most favorable for the cell growth. Under this condition, 3.0 g/L dry cell weight was achieved after a cultivation of 12 h.

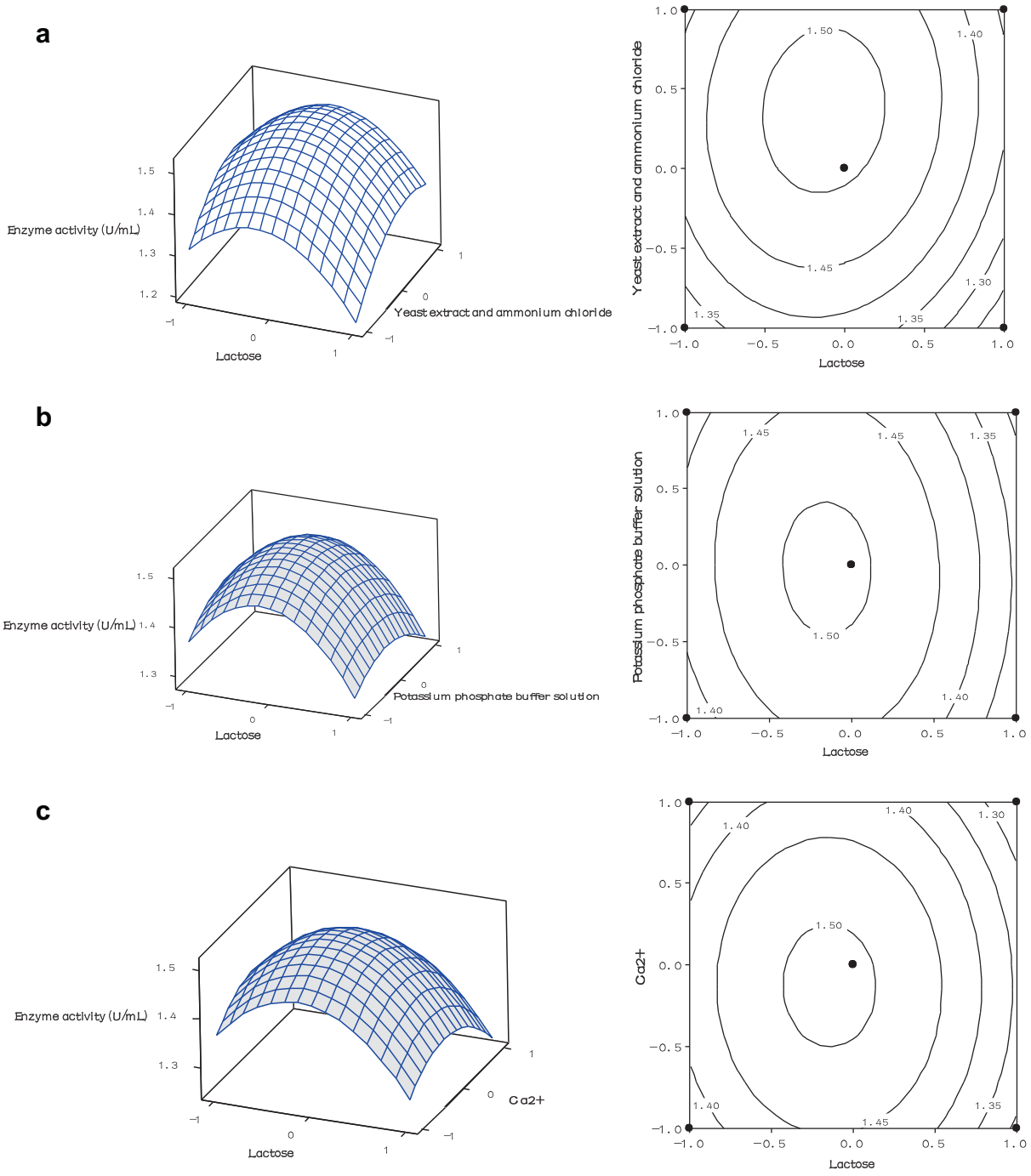

Fig. 6. Response surface plot and the corresponding contour concerning effects on enzyme activity. (a) Lactose and yeast extract/ammonium chloride concentration. (b) Lactose and potassium phosphate buffer solution concentration. (c) Lactose and Ca^{2+} concentration.

3.4. Effect of carbon sources on the biomass yield and enzyme activity

The carbon source in TB medium was replaced by equimolar carbon of dextrin, soluble starch, maltose, citric acid, glucose, lactose and sucrose, respectively. From the obtained results (Fig. 3), enzyme activity was the highest (0.76 U/mL) when lactose was employed as the carbon source. But when glucose was employed as the carbon source, the cell concentration was the highest, and the enzyme activity was much lower than that of lactose. So the lactose was selected as the carbon source.

Different lactose concentrations, ranging from 0 to 20.0 g/L, were used to culture the strain. The results showed that the biomass yield was the highest (3.95 g/L) when the concentration of lactose was 10.0 g/L and the enzyme production reached the highest (0.71 U/mL) when the concentration of lactose was 5.0 g/L. However, taking the production cost into account, 5 g/L would be favorable and selected as the lactose concentration.

3.5. Effect of nitrogen sources on the biomass yield and enzyme activity

The nitrogen sources, yeast extract and peptone in the initial medium, were replaced by equimolar nitrogen of peptone, yeast extract, beef extract, carbamide, ammonium chloride, ammonium

sulfate, ammonium nitrate and sodium nitrate, respectively, to carry out single factor experiment of nitrogen sources. The result indicated the biomass yields and enzyme activities with organic nitrogen sources were all higher than those with inorganic nitrogen sources. When yeast extract was used, the enzyme activity was highest, reaching 0.85 U/mL, with a maximal biomass yield of 4.50 g/L (Fig. 4). So the yeast extract was selected as the nitrogen source.

Considering the significant effect of nitrogen source on biomass yield and enzyme activity, the effects of different composite nitrogen sources with consistent nitrogen content on biomass yield and enzyme activity were also investigated. The result showed that when the mass-volume concentration of yeast extract and ammonium chloride was 37.0 g/L and 2.0 g/L in the medium, both biomass yield and enzyme activity were the highest, 4.93 g/L and 1.08 U/mL, respectively (Table 1).

Seven concentrations of composite nitrogen sources were tested in the experiments. The results were shown in Table 2. Enzyme activity increased along with the concentrations of yeast extract and ammonium chloride until their concentration reached 46.2 g/L and 2.5 g/L, respectively. So in the next study, 46.2 g/L yeast extract and 2.5 g/L ammonium chloride were selected as the nitrogen source.

3.6. Effect of buffer solution on the biomass yield and enzyme activity

Potassium phosphate buffer solution (170 mM KH_2PO_4, 550 mM K_2HPO_4) in TB medium was replaced by 200 mM sodium citrate buffer solution and 200 mM sodium phosphate buffer solution, each with pH of 6.6, respectively. The results indicated that the pH stabilization function of sodium citrate and sodium phosphate buffer solution was not as good as that of potassium phosphate buffer solution and it also indicated that sodium citrate buffer solution would exert an inhibiting effect on the enzyme activity. When potassium phosphate buffer solution was employed, the corresponding enzyme activity and biomass yield were 1.26 U/mL and 4.83 g/L, respectively.

Different volume of potassium phosphate buffer solution was added to the medium to form buffer concentrations of 50, 100, 150, 200 and 300 mL/L, respectively. The result showed that a buffer concentration of 100 mL/L was favorable for the cell growth and enzyme activity, which was 4.84 g/L and 1.29 U/mL, respectively. When buffer concentration was more than 100 mL/L, there was no significant cells accumulation; when buffer concentration was less than 100 mL/L, there was no pH stabilization function of buffer solution, and the low phosphate content would not meet the cell growth condition.

3.7. Effect of metal ions on biomass yield and enzyme activity

2.5 mM Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Li^{+}, Fe^{2+} and Mg^{2+} were added to the medium, respectively. The results showed that metal ions, Ca^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and Mg^{2+}, exerted a promoting effects on the biomass yield and enzyme activity, among which, Ca^{2+} was the most significant, with biomass yield and enzyme activity of 5.2 g/L and 1.35 U/mL, respectively. However, ions, Co^{2+}, Cu^{2+} and Li^{+}, showed inhibiting effects (Fig. 5).

Different Ca^{2+} concentrations, 0 g/L, 0.04 g/L, 0.1 g/L, and 0.2 g/L, were employed in the study. The result indicated that 0.04 g/L Ca^{2+} was favorable for the biomass yield and enzyme activity, which reached 5.48 g/L and 1.45 U/mL, respectively. The reason of promoting function of Ca^{2+} may be that, on the one hand, Ca^{2+} could change membrane permeability so as to promote mass transfer; on the other hand, Ca^{2+} could increase enzyme stability.

3.8. Optimization of significant variables using response surface methodology

Box–Behnken designing method and response surface methodology were employed in the experiments. According to the results of single-factor research, lactose, yeast extract and ammonium chloride, potassium phosphate buffer solution and Ca^{2+} were taken as the optimum component of medium. A four factors and three levels experiment was designed. The coding levels of factors and experimental results were shown in Table 3.

Statistical software, Minitab, was applied to carry out quadratic polynomial regression fitting. The property of polynomial model equation was expressed by R2 and the coefficient of determination and its statistical significance were determined by the *T* test.

The process of data analysis was conducted through RSREG (response surface), by which the quadratic response surface regression model was established to gain the optimum level of corresponding factor. The analysis results were shown in Table 4.

Ternary quadratic equation of enzyme activity about factors, lactose, yeast extract/ammonium chloride, potassium phosphate buffer solution and Ca^{2+}, was as follows:

$$\begin{aligned} Y = {} & 1.507 - 0.037X_1 + 0.054X_2 - 0.003X_3 - 0.019X_4 - 0.126X_1^2 \\ & - 0.071X_2^2 - 0.049X_3^2 - 0.073X_4^2 + 0.014X_1X_2 - 0.006X_1X_3 \\ & - 0.002X_1X_4 - 0.001X_2X_3 + 0.003X_2X_4 - 0.038X_3X_4 \end{aligned}$$

In the equation, X_1, X_2, X_3 and X_4 represented the coding levels of factors of lactose, yeast extract/ammonium chloride, potassium phosphate buffer solution and Ca^{2+}, respectively. The regression equation analysis of variance test of significance showed that the equation *F* value was greater than $F_{0.01}$, which meant that the lack of fit was insignificant in the model, while the regression was significant. In addition, the model coefficient of determination R2 was 0.9375, indicating that the fitting degree of equation was good, and there was a high degree of correlation between the predicted value and measured value. Thus it could be applied to predict the theoretical enzyme activity.

By solving the equations, the optimum conditions of cultivation could be obtained. When the concentration of lactose, yeast extract/ammonium chloride, potassium phosphate buffer solution and Ca^{2+} was 4.7 g/L, 49.5 g/L, 2.7 g/L, 110 mL/L and 0.0352 g/L, the theoretical highest enzyme activity, 1.52 U/mL, could be obtained.

The relation between factors and enzyme activity was indicated by the surface and contour line (Fig. 6a–c).

3.9. Verification test

After repeated experiments employing the optimized medium, the average actual enzyme activity reached to 1.49 U/mL, and dry cell weight was 5.5 g/L, which was much more significantly higher than that under initial condition. Therefore, the optimization was effective.

4. Conclusions

*val*G from *S. hygroscopicus* was successfully cloned into *E. Coli* BL21(DE3) and was employed in biotransformation from validoxylamine A to validamycin A with the existence of D-cellobiose using the free resting cells. The medium was optimized. The optimized medium was lactose 4.7 g/L, yeast extract 49.5 g/L, NH_4Cl 2.7 g/L, potassium phosphate buffer solution 110 mL/L and Ca^{2+} 0.0352 g/L. With the medium, the biomass yield and enzyme activity reached 5.5 g/L and 1.49 U/mL, respectively, which were nearly twice higher than that with initial medium. Further work should be carried out to accomplish the application in biotransformation.

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