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Short communication

Statistical optimization for production of chitin deacetylase from *Rhodococcus erythropolis* HG05

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ABSTRACT

A strain producing chitin deacetylase (CDA) was isolated and identified as *Rhodococcus erythropolis* by morphological characteristics and 16S rDNA analysis, named as *R. erythropolis* HG05. By Plackett–Burman and central composite design, CDA production from *R. erythropolis* HG05 was increased from 58.00 U/mL to 238.89 U/mL. With the crude enzyme from *R. erythropolis* HG05, the hydrolysate components from colloid chitin were chito-oligosaccharides with polymerization number larger than hexaose.

1. Introduction

At present, chitosan is mainly produced by thermo-alkaline deacetylation of chitin. The process is environmentally unsafe, tedious to control and results into heterogeneous range of products (Chang, Tsai, Lee, & Fu, 1997; Muzzarelli, 2012). Alternatively, chitosan can also be produced through chitin deacetylase (CDA, EC 3.5.1.41), which overcomes most of disadvantages in alkali treatment (Trudel & Asselin, 1990). Some microorganisms were reported to produce CDAs (Dai, Li, Hu, & Sa, 2011; Pareek, Vivekanand, Saroj, Sharma, & Singh, 2012; Zhao, Park, & Muzzarelli, 2010). It seems that CDAs from different sources may have different activity, stability, specificity, and efficiency. CDAs assisted enzymatic conversion to chitosan needs intensive screening of novel CDA hyper-producers.

Medium components and culture conditions have great influence on extracellular enzyme production and are different for each microorganism. Optimization of variables by statistical designs can eliminate the limitations of one-factor-at-a-time approach. The objective of this research was to apply Plackett–Burman design, followed by response surface methodology to optimize medium components and culture conditions for CDA production by *Rhodococcus erythropolis* HG05. In addition, the enzymatic hydrolysate was also analyzed.

2. Materials and methods

2.1. Strain, media and culture conditions

The bacterial strain was isolated from soil samples collected from Lianyungang of China and maintained on Luria Bertani (LB) medium agar slants.

Pre-culture was prepared by growing the strain in LB medium at 30 °C, 165 r/min till strain density reached 3.0 at $OD_{650\ nm}$. Main culture medium consisted (g/L): powder chitin (Sigma C7170) 10.0, $(NH_4)_2SO_4$ 25.0, yeast extract 5.0, glucose 0.5, $MgSO_4 \cdot 7H_2O$ 1.0, $K_2HPO_4 \cdot 3H_2O$ 1.0, KH_2PO_4 0.3, and NaCl 0.5, pH 7.0. Main culture was maintained at the same conditions as pre-culture for 60 h, inoculated with 4% inoculum (V/V) and culture flasks (250 mL) containing 40 mL medium. All experiments were triplicated.

2.2. CDA assay

The supernatant of main culture centrifugated at 10,000 × *g* for 10 min (4 °C) was used in CDA assay and 4-nitroacetanilide was used as the substrate. The reaction mixture, containing 0.1 mL of 200 mg/L 4-nitroacetanilide, 0.1 mL of diluted enzyme solution, and 0.3 mL of 0.2 M phosphate buffer (pH 7.0), was incubated at 50 °C for 15 min. Heated for 5 min in boiling water to inactivate CDA, the mixture was added ddH_2O up to 1 mL. The solution was centrifuged and CDA activity was determined by measuring amount of 4-nitroaniline released from 4-nitroacetanilide at $OD_{400\ nm}$. One unit of CDA is defined as activity that catalyze the release of 1 μg

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Table 1
Range of different variables studied in PBD.

Variables	Names	Levels	
		−1	+1
X_1 (g/L)	Glucose	8.00	12.00
X_2 (g/L)	Powder chitin	7.00	10.00
X_3 (g/L)	$(NH_4)_2SO_4$	20.00	30.00
X_4 (g/L)	Yeast extract	4.00	6.00
X_5 (g/L)	KH_2PO_4	0.35	0.53
X_6 (g/L)	$K_2HPO_4{\cdot}3H_2O$	3.00	4.50
X_7 (g/L)	NaCl	0.40	0.60
X_8 (g/L)	$MgSO_4{\cdot}7H_2O$	1.50	3.25
X_9	pH	7.0	8.0
X_{10} (°C)	Temperature	28	32
X_{11} (h)	Time	60	72
X_{12} (%)	Inoculums size	2.5	3.5
X_{13} (mL/250 mL)	Fermentation holding	35	50

of 4-nitroaniline per hour from 4-nitroacetanilide under standard assay conditions.

2.3. Experimental design and data analysis

2.3.1. Optimization of medium components and culture conditions using Plackett–Burman design (PBD)

Based on the result of optimization of medium components and culture conditions with one-factor-at-a-time method (data not shown), PBD was employed to evaluate the important factors affecting CDA production (Table 1). The statistical software package "Design Expert 8.05b" (Stat-Ease Inc., Minneapolis, USA) was used to generate and analyze experimental design (Desai, Akolkar, Badhe, Tambe, & Lele, 2006). Six dummy variables (X_{14}, X_{15}, X_{16}, X_{17}, X_{18}, and X_{19}) were designated. Concentration ranges for variables were decided by extensive literature survey. Response was determined by CDA activity.

2.3.2. Central composite design (CCD)

Response surface methodology (RSM) was used to determine optimum value of selected relevant variables for elevation of CDA production. A CCD was used to optimize variables and experimental design was formulated and analyzed by "Design Expert 8.05b". Response was determined by CDA activity.

2.4. Determination of the degree of deacetylation (DDA) of chitin

The DDA of chitin was measured by the acid hydrolysis-HPLC method (Ng, Hein, Chandrkrachang, & Stevens, 2006).

2.5. Analysis of hydrolysate components

Culture was centrifuged at 14,000 × g for 30 min (4 °C). The supernatant was added into 5% colloidal chitin, stirred evenly, undertaken vibration reaction at 50 °C for 24 h. The enzymatic product was centrifuged and qualitatively analyzed by thin layer chromatography (Choi, Kim, Piao, Yun, & Shin, 2004).

3. Results and discussion

3.1. Isolation, screening and identification of a CDA-producing strain

Among 23 isolated bacterial species, a strain with maximum extracellular CDA activity (58.00 U/mL) was selected for further studies. It also had a considerable level of chitinase activity. Based on morphological characteristics and 16S rDNA analysis (Accession no.: KF439699), it was identified as *R. erythropolis* and named *R. erythropolis* HG05.

Table 2
Identification of significant variables for CDA production by *R. erythropolis* HG05 using PBD.

Source	Sum of squares	*DF*	Mean square	*F*-value	Prob > *F*
Model	10,510.21	13	808.48	17.99	0.0010[a]
X_1	2102.89	1	2102.89	46.79	0.0005[a]
X_2	13.51	1	13.51	0.30	0.6032
X_3	45.30	1	45.30	1.01	0.3541
X_4	8.48	1	8.48	0.19	0.6793
X_5	39.03	1	39.03	0.87	0.3873
X_6	37.65	1	37.65	0.84	0.3954
X_7	3119.50	1	3119.50	69.41	0.0002[a]
X_8	1255.80	1	1255.80	27.94	0.0019[a]
X_9	60.55	1	60.55	1.35	0.2898
X_{10}	16.34	1	16.34	0.36	0.5686
X_{11}	5.81	1	5.81	0.13	0.7315
X_{12}	3701.38	1	3701.38	82.36	0.0001[a]
X_{13}	103.97	1	103.97	2.31	0.1791
Residual	269.65	6	44.94		
Cor total	10,779.86	19			

[a] Significant at 1% level.

3.2. Plackett–Burman design

Thirteen-factor-twenty-run experiments were conducted and a significant variation in CDA production was observed (7.89–93.21 U/mL) that highlighted the importance of optimizing variables to attain higher enzyme activity. The regression analysis made it clear that glucose, NaCl, $MgSO_4$, and inoculums size had the most significant influence on CDA production (Table 2).

3.3. Response surface methodology

Thirty experiments were performed using Box–behnken design of RSM (Table 3). The results were analyzed by standard

Table 3
Experimental design matrix for optimization of CDA production.

Runs	X_1	X_7	X_8	X_{12}	CDA activity (U/mL)	
					Observed	Predicted
1	10.00	0.6	4.13	3.50	84.00	87.64
2	9.00	0.5	2.38	3.00	83.32	90.18
3	9.00	0.5	0.63	3.00	143.32	135.84
4	8.00	0.4	1.50	2.50	82.56	82.28
5	8.00	0.6	1.50	2.50	98.28	99.73
6	8.00	0.4	3.25	3.50	85.13	82.27
7	9.00	0.5	4.13	3.00	93.85	98.14
8	10.00	0.4	1.50	3.50	105.13	104.60
9	9.00	0.5	2.38	3.00	104.42	109.59
10	11.00	0.5	2.38	3.00	90.99	87.64
11	9.00	0.5	2.38	3.00	84.85	80.44
12	9.00	0.5	2.38	2.00	96.71	97.86
13	8.00	0.6	3.25	2.50	90.28	87.64
14	9.00	0.5	2.38	3.00	83.42	87.64
15	8.00	0.4	3.25	2.50	77.43	86.81
16	10.00	0.4	3.25	3.50	89.42	87.64
17	9.00	0.5	2.38	4.00	85.99	82.91
18	9.00	0.5	2.38	3.00	101.85	108.14
19	10.00	0.6	1.50	2.50	93.71	95.75
20	10.00	0.5	1.50	2.50	88.56	87.70
21	10.00	0.6	1.50	3.50	69.99	70.03
22	8.00	0.6	1.50	3.50	102.28	99.33
23	10.00	0.6	3.25	2.50	84.28	81.33
24	9.00	0.7	2.38	3.00	77.71	80.27
25	7.00	0.5	2.38	3.00	92.42	86.67
26	8.00	0.6	3.25	3.50	91.13	92.29
27	9.00	0.3	2.38	3.00	120.99	119.54
28	8.00	0.4	1.50	3.50	103.71	100.63
29	10.00	0.4	3.25	2.50	95.13	99.24
30	9.00	0.5	2.38	3.00	120.85	121.41