

## Short communication

Statistical optimization for production of chitin deacetylase from *Rhodococcus erythropolis* HG05Yuying Sun<sup>a,b</sup>, Jiquan Zhang<sup>b,\*</sup>, Shengjun Wu<sup>a</sup>, Shujun Wang<sup>a</sup><sup>a</sup> Jiangsu Key Laboratory of Marine Biotechnology, Huaihai Institute of Technology, 59 Cangwu Road, Lianyungang 222005, China<sup>b</sup> Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, China

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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.

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## 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<sub>650 nm</sub>. Main culture medium consisted (g/L): powder chitin (Sigma C7170) 10.0, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 25.0, yeast extract 5.0, glucose 0.5, MgSO<sub>4</sub>·7H<sub>2</sub>O 1.0, K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O 1.0, KH<sub>2</sub>PO<sub>4</sub> 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 centrifuged 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<sub>2</sub>O 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<sub>400 nm</sub>. 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 <sub>1</sub> (g/L)        | Glucose                                            | 8.00   | 12.00 |
| X <sub>2</sub> (g/L)        | Powder chitin                                      | 7.00   | 10.00 |
| X <sub>3</sub> (g/L)        | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>    | 20.00  | 30.00 |
| X <sub>4</sub> (g/L)        | Yeast extract                                      | 4.00   | 6.00  |
| X <sub>5</sub> (g/L)        | KH <sub>2</sub> PO <sub>4</sub>                    | 0.35   | 0.53  |
| X <sub>6</sub> (g/L)        | K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O | 3.00   | 4.50  |
| X <sub>7</sub> (g/L)        | NaCl                                               | 0.40   | 0.60  |
| X <sub>8</sub> (g/L)        | MgSO <sub>4</sub> ·7H <sub>2</sub> O               | 1.50   | 3.25  |
| X <sub>9</sub>              | pH                                                 | 7.0    | 8.0   |
| X <sub>10</sub> (°C)        | Temperature                                        | 28     | 32    |
| X <sub>11</sub> (h)         | Time                                               | 60     | 72    |
| X <sub>12</sub> (%)         | Inoculums size                                     | 2.5    | 3.5   |
| X <sub>13</sub> (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<sub>14</sub>, X<sub>15</sub>, X<sub>16</sub>, X<sub>17</sub>, X<sub>18</sub>, and X<sub>19</sub>) 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, Chandkrachang, & 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

**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 <sup>a</sup> |
| X <sub>1</sub>  | 2102.89        | 1  | 2102.89     | 46.79   | 0.0005 <sup>a</sup> |
| X <sub>2</sub>  | 13.51          | 1  | 13.51       | 0.30    | 0.6032              |
| X <sub>3</sub>  | 45.30          | 1  | 45.30       | 1.01    | 0.3541              |
| X <sub>4</sub>  | 8.48           | 1  | 8.48        | 0.19    | 0.6793              |
| X <sub>5</sub>  | 39.03          | 1  | 39.03       | 0.87    | 0.3873              |
| X <sub>6</sub>  | 37.65          | 1  | 37.65       | 0.84    | 0.3954              |
| X <sub>7</sub>  | 3119.50        | 1  | 3119.50     | 69.41   | 0.0002 <sup>a</sup> |
| X <sub>8</sub>  | 1255.80        | 1  | 1255.80     | 27.94   | 0.0019 <sup>a</sup> |
| X <sub>9</sub>  | 60.55          | 1  | 60.55       | 1.35    | 0.2898              |
| X <sub>10</sub> | 16.34          | 1  | 16.34       | 0.36    | 0.5686              |
| X <sub>11</sub> | 5.81           | 1  | 5.81        | 0.13    | 0.7315              |
| X <sub>12</sub> | 3701.38        | 1  | 3701.38     | 82.36   | 0.0001 <sup>a</sup> |
| X <sub>13</sub> | 103.97         | 1  | 103.97      | 2.31    | 0.1791              |
| Residual        | 269.65         | 6  | 44.94       |         |                     |
| Cor total       | 10,779.86      | 19 |             |         |                     |

<sup>a</sup> Significant at 1% level.

no.: KF439699), it was identified as *R. erythropolis* and named *R. erythropolis* HG05.

### 3.2. Plackett–Burman design

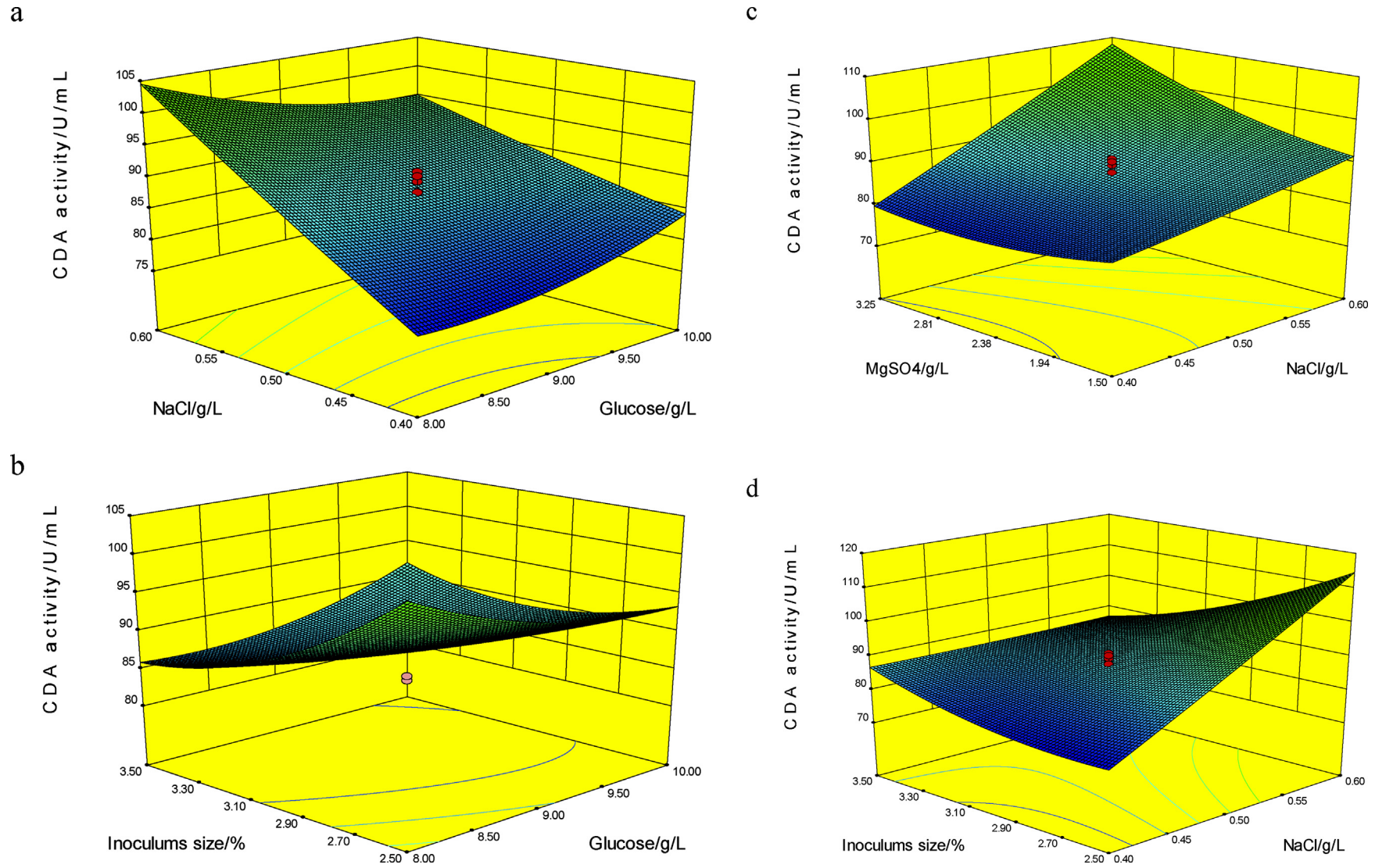
Thirteen-factor-twenty-run experiments were conducted and a significant variation in CDA production was observed (7.89–93.21 U/mL) that highlighted the importance of optimizing variables to attain higher enzyme activity. The regression analysis made it clear that glucose, NaCl, MgSO<sub>4</sub>, and inoculums size had the most significant influence on CDA production (Table 2).

### 3.3. Response surface methodology

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

**Table 3**  
Experimental design matrix for optimization of CDA production.

| Runs | X <sub>1</sub> | X <sub>7</sub> | X <sub>8</sub> | X <sub>12</sub> | 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    |



**Fig. 1.** Response surface plots and its contour plot of CDA production by *R. erythropolis* HG05 optimization of variables. The interaction between (a) glucose and NaCl, (b) glucose and inoculum size, (c) NaCl and MgSO<sub>4</sub>, and (d) NaCl and inoculum size.

**Table 4**  
Multiple regression analysis of CCD.

| Sources                        | Sum of squares | DF | Mean square | F-value | Prob > F             |
|--------------------------------|----------------|----|-------------|---------|----------------------|
| Model                          | 5731.37        | 14 | 409.38      | 16.46   | <0.0001 <sup>a</sup> |
| X <sub>1</sub>                 | 8.53           | 1  | 8.53        | 0.34    | 0.5668               |
| X <sub>7</sub>                 | 2177.61        | 1  | 2177.61     | 87.53   | <0.0001 <sup>a</sup> |
| X <sub>8</sub>                 | 311.98         | 1  | 311.98      | 12.54   | 0.0030 <sup>a</sup>  |
| X <sub>12</sub>                | 448.68         | 1  | 448.68      | 18.04   | 0.0007 <sup>a</sup>  |
| X <sub>1</sub> X <sub>7</sub>  | 270.19         | 1  | 270.19      | 10.86   | 0.0049 <sup>a</sup>  |
| X <sub>1</sub> X <sub>8</sub>  | 20.14          | 1  | 20.14       | 0.81    | 0.3825               |
| X <sub>1</sub> X <sub>12</sub> | 210.61         | 1  | 210.61      | 8.47    | 0.0108 <sup>b</sup>  |
| X <sub>7</sub> X <sub>8</sub>  | 358.63         | 1  | 358.63      | 14.42   | 0.0018 <sup>a</sup>  |
| X <sub>7</sub> X <sub>12</sub> | 1418.09        | 1  | 1418.09     | 57.00   | <0.0001 <sup>a</sup> |
| X <sub>8</sub> X <sub>12</sub> | 13.86          | 1  | 13.86       | 0.56    | 0.4670               |
| X <sub>1</sub> <sup>2</sup>    | 148.55         | 1  | 148.55      | 5.97    | 0.0274 <sup>b</sup>  |
| X <sub>7</sub> <sup>2</sup>    | 3.60           | 1  | 3.60        | 0.14    | 0.7091               |
| X <sub>8</sub> <sup>2</sup>    | 163.09         | 1  | 163.09      | 6.56    | 0.0217 <sup>b</sup>  |
| X <sub>12</sub> <sup>2</sup>   | 303.41         | 1  | 303.41      | 12.20   | 0.0033 <sup>a</sup>  |
| Residual                       | 373.17         | 15 | 24.88       |         |                      |
| Lack of fit                    | 320.75         | 10 | 32.07       | 3.06    | 0.1145               |
| Pure error                     | 52.42          | 5  | 10.48       |         |                      |
| Cor total                      | 6104.54        | 29 |             |         |                      |

<sup>a</sup> Significant at 1% level.

<sup>b</sup> Significant at 5% level.

analysis of variance (ANOVA) and quadratic regression equations were obtained as follows:

$$Y = 87.64 - 0.60X_1 + 9.53X_7 + 3.61X_8 - 4.32X_{12} - 4.11X_1X_7 + 1.12X_1X_8 + 3.63X_1X_{12} + 4.73X_7X_8 - 9.41X_7X_{12} - 0.93X_8X_{12} + 2.33X_1^2 + 0.36X_7^2 + 2.44X_8^2 + 3.33X_{12}^2$$

where Y represents CDA activity.

As analysis of this model, X<sub>7</sub>, X<sub>8</sub>, X<sub>12</sub>, X<sub>1</sub><sup>2</sup>, X<sub>8</sub><sup>2</sup>, X<sub>12</sub><sup>2</sup>, and interaction effect of X<sub>1</sub>X<sub>7</sub>, X<sub>1</sub>X<sub>12</sub>, X<sub>7</sub>X<sub>8</sub>, X<sub>7</sub>X<sub>12</sub> played important role in CDA production (Table 4, Fig. 1).

The results predicted by equation showed that combination of glucose 7.00 g/L, NaCl 0.70 g/L, MgSO<sub>4</sub> 4.13 g/L, and inoculums size 2% would favor CDA production, giving 244.45 U/mL. Under the predicted optimal conditions, CDA production reached 238.89 U/mL by experiment and increased 3.12-fold compared with that under original conditions. When colloidal chitin with 40.25% DDA was used as single carbon source in culture medium, DDA of chitin was 49.89% after fermentation, which showed that CDA from *R. erythropolis* HG05 could deacetylate chitin.

#### 3.4. Analysis of hydrolysate components

Chito-oligosaccharides (COSs) can be prepared by CDA and chitinase. At present, one-step hydrolysis of chitin to COSs by crude

enzymes was only reported by Dai et al. (2011) and its enzymatic hydrolysates were mainly COSs with polymerization number less than hexaose. However, the polymerization number of COSs produced by crude enzyme from *R. erythropolis* HG05 were larger than hexaose (data not shown).

#### 4. Conclusions

In this paper, we isolated and firstly reported *R. erythropolis* HG05 which could produce CDA and chitinase. By statistical optimization of medium components and culture conditions, CDA activity increased by 3.12-fold compared with initial activity. Enzymatic hydrolysates of chitin by crude enzyme were mainly COSs with polymerization number larger than hexaose.

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