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# Optimization of Endoglucanase Production from a Novel Bacterial Isolate, *Arthrobacter* sp. HPG166 and Characterization of Its Properties

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## ABSTRACT

*In this study, a potential novel cellulolytic bacterium* Arthrobacter *sp. HPG166 was isolated from the hindgut of root-feeding larvae* Holotrichia parallela. *Optimization of fermentation factors for endoglucanase production by* Arthrobacter *sp. HPG166 was carried out via response surface methodology. Sodium carboxymethylcellulose 1.19% (w/v) and beef extract 0.35% (w/v) were the ideal combination of carbon and nitrogen sources for enzyme production; the optimum temperature and pH for cellulase production were 34°C and pH 8.0 respectively. Under the optimized fermentation conditions, the maximum endoglucanase activity of 1.411 U* $mL^{-1}$ *was obtained. The crude endoglucanase was thermotolerant as it retained 50.31% of its activity after incubation at 70°C for an hour. Metal profile of the enzyme indicated that* $Mg^{2+}$ *and* $Na^{+}$ *were strong stimulators while* $Mn^{2+}$ *and* $Co^{+}$ *drastically inhibited its activity. Due to its particular characteristics, this enzyme could have potential for industrial applications.*

**Key words:** Scarab larvae, Cellulolytic bacteria, Response surface methodology, Thermotolerant cellulases, Effects of Metal ions

## INTRODUCTION

In the recent years, growing attention has been devoted to the cellulases because of the diversity of their applications such as paper recycling, laundry, cotton processing, biofuel production, food processing, as well as pharmaceutical applications (Pradeep and Narasimha 2011; Acharya and Chaudhary 2012). Since the cellulose enzymes are used in a wide variety of industrial applications, the demand for more stable, highly active, and substrate specific enzymes is also growing rapidly (Shahzadi et al. 2014). Cellulases are inducible enzymes and synthesized by a large diversity of microorganisms, including fungi and bacteria (Abdel-Fatah et al. 2012). Accordingly, it is highly desirable to exploit new organisms capable of producing highly efficient cellulolytic enzymes and determine the most efficient method for producing these enzymes.

There has been large emphasis on the use of fungi because of their capability to produce large amounts of cellulases and hemicellulases, which are secreted into the medium for easy extraction and purification (Maki et al. 2009). Among them, the enzymes produced by *Aspergillus* sp. (Begum and Alimon 2011) and *Trichoderma* sp. (Li et al. 2010) have been extensively studied and used as

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commercial cellulases. In the bioprocess and modern industrial applications, the enzymes often need to have some unique properties, such as high thermal stability, salt-tolerance, pH stability, heavy metal tolerance, and resistance to acid or alkaline media in extreme conditions. However, a major disadvantage for industrial application is that most cellulases of fungal origin lack thermal stability at high temperature (Acharya and Chaudhary 2012). Since most industrial processes are carried out at high temperatures, the industrial applications of fungal origin cellulases are correspondingly limited (Acharya and Chaudhary 2011). Bacteria have high growth rate and enzyme production rate as compared to fungi and have good potential to be used in cellulase production (Ladeira et al. 2015). More importantly, bacterial cellulases can survive in harsh conditions of bioconversion processes, and also be less inhibited by the presence of material that has already been hydrolyzed (feedback inhibition) (Kadarmoidheen et al. 2012). Therefore, the isolation and characterization of novel glycoside hydrolases from bacteria are now becoming widely exploited (Maki et al. 2009; Abdel-Fatah et al. 2012), and the cellulolytic property of some bacterial genera such as *Clostridium* (Yuan et al. 2015), *Cellulomonas* (Kojima et al. 2013), *Pseudomonas* (Huang et al. 2012; Sheng et al. 2012), *Bacillus* (Gaur and Tiwari 2015), and *Streptomyces* (Azzeddine et al. 2013) has been widely reported.

*Athrobacter* species are predominant members of culturable soil bacteria and can utilize a wide variety of organic compounds, such as aromatic hydrocarbons, nucleic acids and their degradation products, herbicides (Crocker et al. 2000). However, there have been rare reports that strains of this species could be cellulolytic (Jones and Keddie 2006; Soares et al. 2012). In this study, a potential novel cellulolytic bacterium *Arthrobacter* sp. HPG166 was isolated from the hindgut of root-feeding larvae, *Holotrichia parallela*. The culture condition of the bacterium was optimized and the characteristics of the enzyme were also studied.

## MATERIAL AND METHODS

### Insect and Dissection

Third-instar larvae of *H. parallela* were collected from a peanut field in Xuzhou, Jiangsu Province and dissected as described by Huang et al. (2012). Then, each hindgut (from the pyloric sphincter to the rectum including the modified fermentation sac) was placed into a 1.5 mL eppendorf tube, which contained 0.5 mL of phosphate buffered saline. The hindgut sections of eight individual larvae were pooled and homogenized, and the suspension was well mixed.

### Isolation of Cellulolytic Bacteria

The suspension of the homogenate (100 μL) was incubated aerobically in 100 mL sodium carboxymethylcellulose (CMC) medium containing (g/L), 10 CMC (low viscosity, Sigma), 5.0 beef extract, 4.0 $KH_2PO_4$, 4.0 $Na_2HPO_4$, 0.2g $MgSO_4 \cdot 7H_2O$, 0.5g NaCl, 0.001g $CaCl_2 \cdot 2H_2O$, 0.004g $FeSO_4 \cdot 7H_2O$ at 30 ± 1°C. After three weeks, the culture was serially diluted ten-fold (to $10^{-9}$). For each dilution, 100 μL was spread on solid CMC medium and aerobically incubated at 30 ± 1°C for six days. Bacterial isolates from single colonies were repeatedly grown on solid agar plates until a pure culture was obtained. Pure isolates were maintained at 4°C in a refrigerator for further studies.

### Screening of Cellulase Producing Bacteria

The activity of endoglucanase production of the isolates was tested on solid CMC medium by covering the Petri dishes with congo red dye, as described by Teather and Wood (1982). The hydrolysis capacity (HC), that is, the ratio of the clear zone diameter to the colony diameter, was employed to represent the preliminary enzyme production abilities of these isolates. The production of endoglucanase by the isolates was determined by the dinitro salicylic acid (DNS) method (Ghose 1987). Finally, the isolate HPG166 with the highest cellulase-producing activity was chosen for further studies.

### 16S rRNA Gene Sequencing and Phylogenetic Analysis

Chromosomal DNA was extracted from the strain HPG166 with TIANamp Bacteria DNA Kit (TIANGEN Biotech Co., Ltd., Beijing, China). Then DNA fragment of 16S rRNA gene was amplified as previously described in Huang et al. (2012). The PCR product was cloned into *pE*ASY-*T1* cloning kit (Beijing TransGen Biotech Co., Ltd., Beijing, China) with blue-white screening. The clones containing inserts of the correct size were sequenced, and the sequences were aligned against those found in the NCBI database and on the EzTaxon-e server (Kim et al. 2012).

Phylogenetic analysis of strain HPG166 was performed by MEGA4.0 (Kumar et al. 2008). The Maximum Likelihood (ML) tree was constructed from the distance matrix calculated by the algorithm of Kimura's two-parameter model. Bootstrap confidence values were obtained with 1000 re-samplings. The sequence has been submitted to the GenBank database under accession number KF887413.

**Morphological, Physiological and Biochemical Characterization**

The morphological, physiological and biochemical characteristics of the isolate was performed according to the standard methods (Smibert and Krieg 1994). The standard physical and biochemical tests, including motility, Gram staining, methyl red (MR) test, Voges-Proskauer (VP) test, activities of catalase, oxidase, urease, production of indole, and utilization of citrate were performed. Different carbon sources (citrate, inositol, D-lactose, D-glucose, D-maltose, D-mannitol, D-arabitol, erythritol, rhamnose, and β-methyl-xyloside) were used to evaluate carbon utilization. Except for the gelatinase activity test (which was performed at 20°C), all of the tests were performed at 28°C in the appropriate medium and were conducted according to standard methods.

**Optimization of Culture Conditions for Endoglucanase Production**

To determine the effect of carbon/nitrogen sources and culture condition on the endoglucanase production by the bacterial isolate, response surface methodology (RSM) with central composite rotatable design (CCRD) was engaged to screen and determine the optimum levels of the significant parameters. The effects of the four independent variables, CMC concentration, beef extract concentration, pH and temperature were studied at five coded levels (-2,-1,0,+1,+2). The ranges and levels of the variables used in experimental design matrix are presented in Tables 1 and 2. Endoglucanase activity ($UmL^{-1}$) was the dependent variable. The center point was repeated six times to determine the repeatability of the method. The applied design was integrated to determine the combined effect of the independent variables and each response and find the optimum level of the independent variables resulting in the endoglucanase production. Cultivations were performed in 250 mL Erlenmeyer flasks containing 100 mL of base medium. All of the flasks were incubated at 180 rpm for 24 h. After cultivation, the fermented broth was centrifuged at 5,000×g, 4°C for 10 min, and the cell-free supernatant (crude enzyme preparation) was used for endoglucanase activity determination.

**Table 1** - Levels of variables tested in CCRD.*

| Variables | | Coded levels | | | | |
|---|---|---|---|---|---|---|
| | | -2 | -1 | 0 | +1 | +2 |
| $X_1$ | CMC (%) | 0.2 | 0.6 | 1.0 | 1.4 | 1.8 |
| $X_2$ | Beef Extract (%) | 0.05 | 0.15 | 0.25 | 0.35 | 0.45 |
| $X_3$ | Temperature (°C) | 24 | 27 | 30 | 33 | 36 |
| $X_4$ | pH | 5 | 6 | 7 | 8 | 9 |

*CCRD: central composite rotatable design, CMC: sodium carboxymethylcellulose.

**Standard Endoglucanase Activity assay**

Endoglucanase activity was determined by the DNS method (Ghose 1987) by measuring the amount of reducing sugars (glucose) in a reaction mixture, which contained 0.5 mL of the crude enzyme sample (cell-free supernatant) and 1.0 mL of 1.0% (w/v) CMC solution. The test tube was incubated at 50°C for 30 min, then 3.0 mL of 3,5-dinitrosalicylic acid was added and boiled for 5 min. Finally, the absorbance at 540 nm was measured. One International Unit (IU) of endoglucanase activity was defined as the amount of enzyme that produced 1 μmol glucose equivalent per milliliter per minute under the assay conditions.

**Enzymatic Property Analysis**

To study the effect of pH and temperature on the crude enzyme activity, experiments were carried out at different temperatures (30, 35, 40, 45, 50 and 55°C) and pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0). Then, the enzyme activities in bacterial cultures were assayed by DNS method as described above.

To investigate the effect of ions on enzymatic activity, different metal salts were added into the test tubes at 2 mM final concentration. The metal salts involved in the experiment included NaCl, $CaCl_2$, KCl, $BaCl_2$, $CuSO_4$, $MgSO_4$, $CoSO_4$, $MnSO_4$ and $Fe_2(SO_4)_3$. The enzyme activity assay was done by DNS method as described above. The thermal stability of endoglucanase was tested by pre-incubating the enzyme at 50, 55, 60, 65, 70 or

75°C for 1 h, and then the treated samples were immediately cooled on ice. The residual endoglucanase activity was measured using the DNS method described above.

**Statistical Analysis**

All the experiments were carried out in triplicate. The statistical software package Design-Expert (trial version 7.1.6, Stat-Ease Inc., Minneapolis, MN, USA) was used for the experimental design matrix, regression analysis of the experimental data, and optimization procedure. The fitted polynomial equation was then expressed in the form of three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones.

Treatment effect was analyzed using the analysis of variance (ANOVA) and Duncan multiple range test (SPSS version 16, SPSS Inc., Chicago, IL, USA). Differences were considered to be significant at $p<0.05$.

**Table 2** - CCRD-RSM consisting of experiments for the study of four experimental factors in coded and natural units with experimental results.*

| Runs | Coded values | | | | Endoglucanase (U mL$^{-1}$) | |
|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Act value | Pre value |
| 1 | 0 | 0 | 2 | 0 | 1.292 | 1.306 |
| 2 | 0 | 0 | 0 | 0 | 1.117 | 1.129 |
| 3 | 0 | 0 | 0 | -2 | 1.255 | 1.235 |
| 4 | 1 | 1 | 1 | 1 | 1.342 | 1.340 |
| 5 | 0 | 0 | -2 | 0 | 1.153 | 1.137 |
| 6 | -1 | -1 | -1 | -1 | 1.236 | 1.234 |
| 7 | -1 | -1 | 1 | -1 | 1.232 | 1.258 |
| 8 | 0 | 0 | 0 | 2 | 1.307 | 1.326 |
| 9 | -1 | -1 | -1 | -1 | 1.101 | 1.104 |
| 10 | -1 | -1 | 1 | 1 | 1.259 | 1.253 |
| 11 | 1 | 1 | 1 | 1 | 1.392 | 1.394 |
| 12 | -1 | -1 | 1 | 1 | 1.315 | 1.288 |
| 13 | 0 | 0 | 0 | 0 | 1.326 | 1.313 |
| 14 | -2 | -2 | 0 | 0 | 1.196 | 1.193 |
| 15 | 2 | 2 | 0 | 0 | 1.326 | 1.327 |
| 16 | 0 | 0 | 0 | 0 | 1.341 | 1.340 |
| 17 | 0 | 0 | 0 | 0 | 1.341 | 1.340 |
| 18 | 0 | 0 | 0 | 0 | 1.341 | 1.340 |
| 19 | 1 | 1 | -1 | 1 | 1.215 | 1.194 |
| 20 | 0 | 0 | 0 | 0 | 1.341 | 1.340 |
| 21 | 1 | 1 | -1 | -1 | 1.109 | 1.133 |
| 22 | -1 | -1 | -1 | 1 | 1.153 | 1.151 |
| 23 | -1 | -1 | 1 | -1 | 1.181 | 1.176 |
| 24 | 0 | 0 | 0 | 0 | 1.341 | 1.340 |
| 25 | 1 | 1 | -1 | 1 | 1.294 | 1.295 |
| 26 | 0 | 0 | 0 | 0 | 1.341 | 1.340 |
| 27 | -1 | -1 | -1 | 1 | 1.215 | 1.233 |
| 28 | 1 | 1 | 1 | -1 | 1.262 | 1.248 |
| 29 | 1 | 1 | -1 | -1 | 1.272 | 1.282 |
| 30 | 1 | 1 | 1 | -1 | 1.352 | 1.350 |

*X1: carboxymethylcellulose; X2, Beef Extract;X3, Temperature; X4,pH; Act value, actual value; Pre value, predictive value

## RESULTS AND DISCUSSION

### Isolation and Identification of the Cellulolytic Bacteria

In this study, 121 bacterial strains were isolated among which 53 bacterial isolates showed endoglucanase producing activity. Among all the cellulolytic isolates, the isolate HPG166 showed the highest hydrolytic capacity (Fig. 1), indicating its potential as high endoglucanaseproducing isolate. Further screening using the DNSA method also revealed that it had the highest endoglucanase activity (0.804 $UmL^{-1}$) among all the isolates. This isolate was then selected for further study.

**Figure 1-** Morphological characteristics and endoglucanase activity of isolate HPG 166. (A) Morphological characteristics of isolate HPG 166 grown on LB medium at 30°C. (B) Zone of clearance on cellulose Congo Red agar plates for isolate HPG 166 after 48 h of incubation. A zone of clearance surrounding the colony is indicative of carboxymethylcellulose (CMC) hydrolysis by secreted endoglucanase.

The morphological, physiological and biochemical properties of isolate HPG166 are presented in Table 3, which showed that this isolate had the general biochemical and physiological properties features of the genera *Arthrobacter*. The identification of the isolate HPG166 was further corroborated by its 16S rRNA gene sequence. A phylogenetic tree was constructed through 16S rRNA gene as indicated in Figure 2. The results showed that the isolate HPG166 was phylogenetically related to the members of the genus *Arthrobacter*, and exhibited a maximum similarity of 99% with *A. woluwensis* DSM 10495(T). Hence, this isolate was referred as *Arthrobacter* sp. HPG166.

**Table 3** - physiological and biochemical characteristic of the bacterial isolate HPG 166.

| Characteristic | Result | Characteristic | Result |
|---|---|---|---|
| Gram-staining | + | Assimilation of | |
| Motility | + | Citrate | + |
| Catalase | + | inositol | + |
| Oxidase | - | Glucose | + |
| MR test | - | Lactose | + |
| V-P test | - | Maltose | + |
| Indole test | - | D-arabitol | + |
| Nitrate reduction | - | Mannitol | + |
| Urease | + | erythritol | - |
| Hydrolysis of | | rhamnose | - |
| Aesculin | - | β-methyl-xyloside | - |
| Gelatin | + | Growth in 5% NaCl | + |
| Growth at 5°C | - | Growth in 10% NaCl | - |
| Growth at 42°C | + | | |

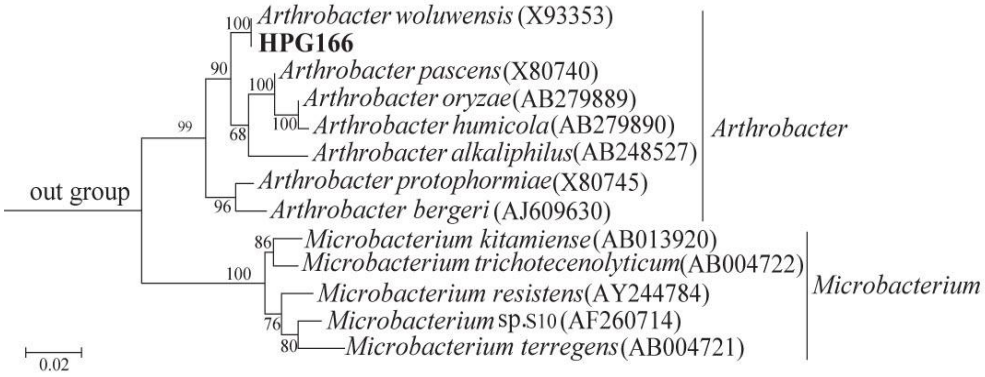


**Figure 2** - Neighbour-joining tree based on the 16S rRNA gene sequence of strain HPG166 and the sequences of representative strains from GenBank. The bar represents 0.02 substitutions per site respectively. *Lactobacillus fermentum* (KC348395) was used as an outgroup.

*Athrobacter* species are widely distributed in the environment and considered to be predominant members of culturable soil bacteria (Crocker et al. 2000). It can utilize a wide variety of organic compounds such as aromatic hydrocarbons, nucleic acids and their degradation products, herbicide, etc. Therefore, *Athrobacter* species were thought to play a significant role in the biodegradation of organic matter (Cacciari and Lippi 1987; Crocker et al. 2000; Jones and Keddie 2006; Niewerth et al. 2012). However, *Arthrobacter* have been rarely reported as cellulose-degrading bacteria (Jones and Keddie 2006; Soares et al. 2012). Furthermore, the close relatives of *Arthrobacter* sp. HPG166 are *Arthrobacter woluwensis*, *Arthrobacter pascens*, and *Arthrobacter oryzae*, but none of these have been reported to possess cellulose degrading capabilities (Funke et al. 1996; Kageyama et al. 2008). Therefore, *Arthrobacter* sp. HPG166 isolated from the hindgut of *H. parallela* larvae could be a potentially novel cellulolytic bacterium of *Arthrobacter* genus.

**Optimization of Culture Conditions for Enzyme Production of *Athrobacter* sp. HPG166**

RSM with CCRD was chosen to optimize four parameters-CMC concentration ($X_1$), beef extract concentration ($X_2$), initial pH of the medium ($X_3$) and temperature ($X_4$) for endoglucanase production of *Arthrobacter* sp. HPG166. As shown in Table 2, the medium constituted in trial 11 gave maximal cellulase production (1.392 U $mL^{-1}$). The derived regression equation for the optimization of medium compounds indicated that the cellulase activity (Y, U=mL) was a combined function of the concentration of CMC ($X_1$), beef extract ($X_2$), pH ($X_3$) and temperature ($X_4$). By analyzing the multiple regressions on the experimental data, the following second-order polynomial equation was derived to clarify the cellulase activity:

$$Y = -1.97021 + 0.64895X_1 + 0.56121X_2 + 0.12230X_3 + 0.20670X_4 - 0.29938X_1X_2 + 0.00408X_1X_3 - 0.02994X_1X_4 + 0.03675X_2X_3 + 0.07650X_2X_4 + 0.00118X_3X_4 - 0.18604X_1^2 - 2.95167X_2^2 - 0.00221X_3^2 - 0.01489X_4^2$$

The analysis of variance (ANOVA) of the model for the endoglucanase yield is shown in Table 4. The predicted models were fitted significantly ($p < 0.0001$). Values of "prob > F" less than 0.05 indicate model terms are significant. In this case, the coefficients $X_1$, $X_3$, $X_4$, $X_1X_2$, $X_1X_4$, $X_2X_3$, $X_1^2$, $X_2^2$, $X_3^2$, and $X_4^2$ were highly significant. The 3D response surface graphs were plotted to better visualize the significant interaction effects of independent variables on the endoglucanase production (Fig. 3). The fit of the model was checked by the coefficient of determination $R^2$. The $R^2$ was calculated as 0.9757, which suggested a marked agreement between the experimentally and theoretically obtained values. The RSM model predicted that a medium containing 1.19% CMC (W/V), 0.35% beef extract (W/V), pH 8.0, and cultured temperature 34°C could give maximum endoglucanase production. Validation of the experimental model was tested by carrying out the experiment under the optimal operation conditions. The maximum enzyme activity predicted by the model was 1.404 $UmL^{-1}$; which was in good agreement with the experimental value 1.411 $UmL^{-1}$; showing 75.5% increase in endoglucanase activity as compared to un-optimised medium. Therefore, the model was reliable for maximizing the production of cellulase by *Athrobacter* sp HPG166.

The production of cellulases is inducible, and is affected by the nature of the substrates and environment in fermentation (Begum and Alimon 2011). The present results showed that CMC had a positive influence on the cellulase production and 1.19% (W/V) CMC was the most effective level. Sheng et al. (2012) found that CMC was the most effective inducer for cellulase production among eight different carbon sources tested, and CMC 1.5% (w/v) led to maximal enzyme yield. Furthermore, an interesting phenomenon was found that the interaction effects of CMC and beef extract had significant effects on cellulase production ($p$=0.0163, Table 4). This result suggested that the C:N ratio of the media could also have an appreciable effect on the cellulase production.

Results showed that the initial pH of medium and temperature had significant impact on the production of endoglucanase; pH 8.0 was the optimum pH for enzyme production, while the optimum temperature was 34°C. Similar results were obtained by Kazemi et al. (2014) whoreported that pH was essential for the production of cellulase by *Bacillus* sp. BCCS A3, while the pH optimum of endoglucanase production was pH 9.0. Sheng et al. (2012) reported that the optimal temperature for endoglucanase production by *Pseudomonas* sp. HP207 was 30°C, while Adeleke (2013) found that

the *Penicillium atrovenetum* exhibited maximum endoglucanase activity at 40ºC. However, the difference of optimal temperature suggested that the optimal temperature for cellulase production also depended on the strain variation of the microorganism.

**Table 4 -** Analysis of Variance (ANOVA) for response surface quadratic model for the production of endoglucanase.

| Source | Sum of squares | DF | Standard error | *F* Value | *P*-value |
|---|---|---|---|---|---|
| Model | 0.19 | 14 | 0.013 | 43.00 | < 0.0001 |
| $X_1$ | $6.323\times10^{-3}$ | 1 | $6.323\times10^{-3}$ | 20.14 | 0.0004 |
| $X_2$ | $2.956\times10^{-4}$ | 1 | $2.956\times10^{-4}$ | 0.94 | 0.3473 |
| $X_3$ | $7.289\times10^{-3}$ | 1 | $7.289\times10^{-3}$ | 23.22 | 0.0002 |
| $X_4$ | $3.005\times10^{-3}$ | 1 | $3.005\times10^{-3}$ | 9.57 | 0.0074 |
| $X_1$* $X_2$ | $2.294\times10^{-3}$ | 1 | $2.294\times10^{-3}$ | 7.31 | 0.0163 |
| $X_1$* $X_3$ | $3.842\times10^{-3}$ | 1 | $3.842\times10^{-3}$ | 1.22 | 0.2861 |
| $X_1$* $X_4$ | $2.294\times10^{-3}$ | 1 | $2.294\times10^{-3}$ | 7.31 | 0.0163 |
| $X_2$* $X_3$ | $1.945\times10^{-3}$ | 1 | $1.945\times10^{-3}$ | 6.20 | 0.0250 |
| $X_2$* $X_4$ | $9.364\times10^{-4}$ | 1 | $9.364\times10^{-4}$ | 0.63 | 0.1047 |
| $X_3$* $X_4$ | $1.988\times10^{-4}$ | 1 | $1.988\times10^{-4}$ | 2.98 | 0.4386 |
| $X_1^2$ | 0.024 | 1 | 0.024 | 77.42 | < 0.0001 |
| $X_2^2$ | 0.024 | 1 | 0.024 | 76.12 | < 0.0001 |
| $X_3^2$ | 0.011 | 1 | 0.011 | 34.57 | < 0.0001 |
| $X_4^2$ | $6.083\times10^{-3}$ | 1 | $6.083\times10^{-3}$ | 19.38 | 0.0005 |
| Residual | $4.709\times10^{-3}$ | 15 | | | |
| Lack of Fit | $4.709\times10^{-3}$ | 10 | | | |
| Pure Error | 0.000 | 5 | | | |
| Cor Total | 0.19 | 29 | | | |

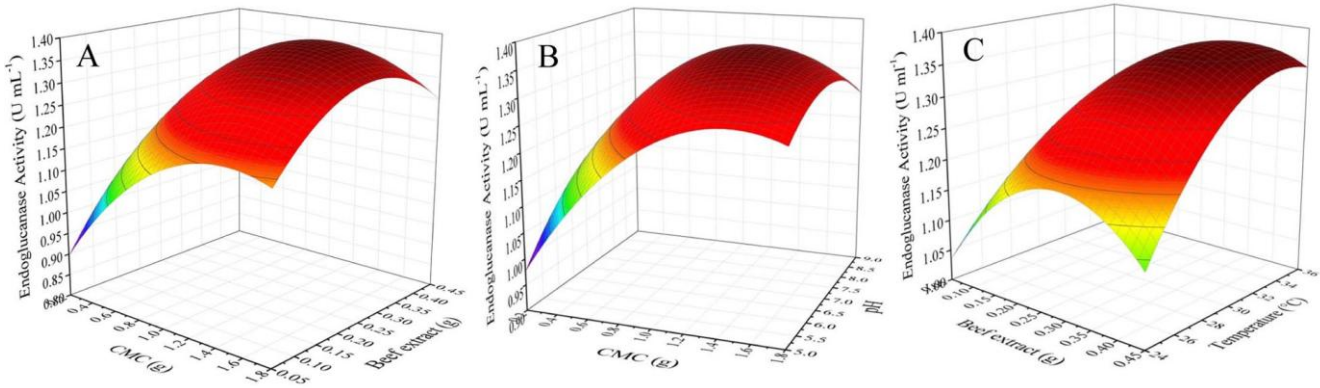


**Figure 3 -** Three-dimensional response surface plot for endoglucanases production showing the interactive effects of different independent variables. (A) carboxymethylcellulose (CMC) and beef extract concentrations as the independent variables. (B) carboxymethylcellulose (CMC) concentration and pH as the independent variables. (C) beef extract concentration and temperature as the independent variables.

**Effect of Temperature, pH, and Metal Ions on Enzymatic Activity**

The endoglucanase activity of *Arthrobacter* sp. HPG166 cellulase was measured at different temperatures (30-55°C) and pH (4.0-10.0). A maximum endoglucanase activity was observed at 50°C (Fig. 4A) and pH 7.0 (Fig. 4B). The thermostability of the enzyme is very important for industrial fermentation. For *Athrobacter*. sp HPG166, the endoglucanase retained nearly full activity after 1 h of incubation at 50 and 55°C (Fig. 4C). Approximately 70.13 and 50.31% of the original enzyme activity were even maintained after pretreatment at 65 and 70°C for 1 h, respectively. Therefore, the enzyme produced by *Athrobacter* sp HPG166 possessed good stability

at a relatively high temperature. Thermostable enzymes, which can hydrolyze lignocellulose to its component sugars, have significant advantages for improving the conversion rate of biomass over their mesophilic counterparts (Wang et al. 2008). Cellulase from *Athrobacter* sp HPG166 had maximum CMC activity at 50°C and showed good thermal stability. The thermostability character enhances the feasibility of further biotechnological exploitation of *Athrobacter*. sp HPG166.

The studies on the effect of several metal ions on endoglucanase activity revealed that the treatment with 2 mM of $Mg^{2+}$, $Na^{+}$ or $Cu^{2+}$ strongly activated the endoglucanase activity of *Athrobacter* sp. HPG166, while $Mn^{2+}$ and $Co^{2+}$ inhibited the enzyme activity (a relative activity of 34.48 and 49.19%, respectively) (Fig.4D). Heavy metals can affect enzyme activities in different ways, such as acting as cofactors for enzymes to promote normal tissue activity, masking the catalytically active groups/enhancing the substrate binding affinity of the enzyme, etc (Giafreda and Bollac 1996). In this study, 2 mM of $Mg^{2+}$ treatment notably improved endoglucanase activity. Similar results were reported by Huang et al. (2013) that 75 mM of $Mg^{2+}$ strongly stimulated endoglucanase activity in *Trichoderma viride* HG 623. Interestingly, the present result showed that $Mn^{2+}$ and $Co^{2+}$ dramatically reduced the endoglucanase activity. However, $Mn^{2+}$ and $Co^{2+}$ were strong activator of cellulase production and activity as found by Franco-Cirigliano et al. (2013). The reasons for these differences are not well understood, but suggest that there could be species effects as well as other environmental factors.

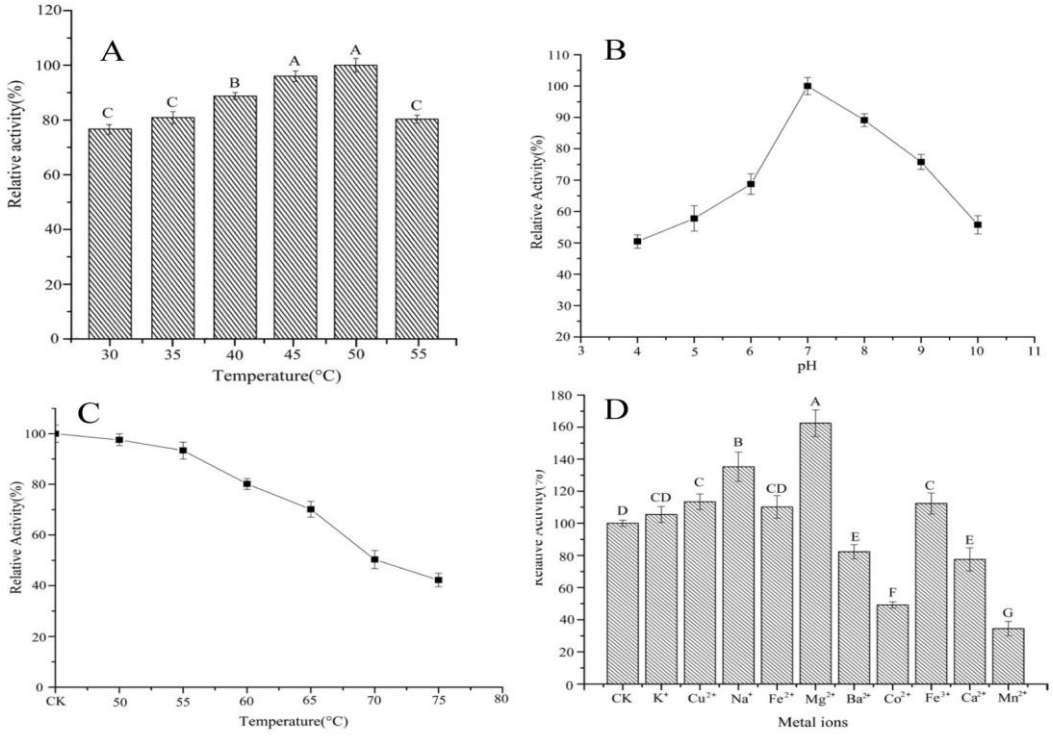


**Figure 4**- Effect of temperature, pH, and metal ions on enzymatic activity. (A) Effects of reaction temperature (30°C to 55°C) on endoglucanase activity. Endoglucanase activities in bacterial cultures at 50°C were considered to be 100%. (B) Effects of reaction pH (4.0 to 10.0) on endoglucanase activity. Endoglucanase activities in bacterial cultures at pH 7.0 were considered to be 100%. (C) Effects of temperature on the stability of endoglucanase. The enzyme activity in bacterial cultures observed after pretreatment at 4°C for 1 h was considered to be 100%. (D) Effects of metal ions on endoglucanase activity. Several different reaction buffers were prepared, each spiked with 2 mM of a metal ion. The enzyme activity of *Arthrobacter* sp. HPG166 that was measured under the normal reaction condition without any additional ions was considered to be 100% (CK). All Values are based on Mean ± SD of three individual observations.

## CONCLUSION

Lignocellulose-degrading insects are attractive potential candidates to prospect for novel cellulolytic microorganisms and enzymes. In this investigation, a potential novel cellulolytic bacterium *Arthrobacter* sp. HPG166 was isolated from the hindgut of scarab beetle larvae. The endoglucanase produced by *Arthrobacter* sp. HPG166 was thermophilic, thermotolerant, and its production could be markedly improved by a number of metal ions, especially $Mg^{2+}$. Therefore, *Athrobacter* sp. HPG166, with features of rapid growth and high endoglucanase production ability, could be a promising strain for biomass degradation in industrial applications.

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## REFERENCES

Abdel-Fatah OM, Hassan MM, Elshafei AM, Haroun BM, Atta HM, Othman AM. Physiological studies on carboxymethyl cellulase formation by *Aspergillus terreus* DSM 826. *Braz J Microbiol*. 2012; 43: 1-11.

Acharya S, Chaudhary A. Effect of nutritional and environmental factors on cellulases activity by thermophilic bacteria isolated from hot spring. *J Sci Ind Res*. 2011; 70: 142-148.

Acharya S, Chaudhary A. Optimization of fermentation conditions for cellulases production by *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3 isolated from Indian hot spring. *Braz Arch Biol Technol*. 2012; 55:497-503 .

Adeleke AJ. Endoglucanase Production by *Penicillium atrovenetum* Using Plantain Peels as Substrate. *AU J T*. 2013; 16:140-146.

Azzeddine B, Abdelaziz M, Estelle C, Mouloud K, Nawel B, Nabila B, et al. Optimization and partial characterization of endoglucanase produced by *Streptomyces* sp. B-PNG23. *Arch Biol Sci Belgrade*. 2013; 65: 549-558.

Begum M, Alimon A. Bioconversion and saccharification of some lignocellulosic wastes by *Aspergillus oryzae* ITCC-4857.01 for fermentable sugar production. *Elect J Biotechnol*. 2011; 14:1-9.

Behera BC, Parida S, Dutta SK, Thatoi HN. Isolation and Identification of Cellulose Degrading Bacteria from Mangrove Soil of Mahanadi River Delta and Their Cellulase Production Ability. *Am J Microbiol Res*. 2014; **2**:41-46.

Cacciari I, Lippi D. Arthrobacters: successful arid soil bacteria. a review. *Arid Soil Res Rehabil*. 1987; 1:1-30.

Crocker FH, Fredrickson JK, White DC, Ringelberg DB, Balkwill DL. Phylogenetic and physiological diversity of *Arthrobacter* strains isolated from unconsolidated subsurface sediments. *Microbiology*. 2000; 146:1295-1310.

Franco-Cirigliano MN, Rezende R C, Gravina-Oliveira MP, Pereira PH, do Nascimento RP, Bon EP, et al. *Streptomyces misionensis* PESB-25 produces a thermoacidophilic endoglucanase using sugarcane bagasse and corn steep liquor as the sole organic substrates. *Biomed Res Int* [Internet]. 2013 [cited 2015 Apr. 29]; **2013**:584207. Available from: http://www.hindawi.com/journals/bmri/2013/584207

Funke G, Hutson RA, Bernard KA, Pfyffer GE, Wauters G, Collins MD. Isolation of *Arthrobacter* spp. from clinical specimens and description of *Arthrobacter cumminsii* sp. nov. and *Arthrobacter woluwensis* sp. nov. *J Clin Microbiol*. 1996; 34: 2356-2363.

Gaur R, Tiwari S. Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. BMC Biotechnol [Internet]. 2015 [cited 2015 Apr. 29]; **15**: 19. Available from: http://www.biomedcentral.com/1472-6750/15/19

Ghose TK. Measurement of cellulase activities. Pure Appl Chem. 1987; **59**: 257-268.

Giafreda L, Bollac JM. Influence of natural an anthropogenic factors on enzyme activity in soil. In Stotzky G, Bollac JM, Eds. Soil biochemistry, New York: Marcel Dekker; 1996. p. 123-193.

Huang SW, Sheng P, Zhang HY. Isolation and Identification of Cellulolytic Bacteria from the Gut of *Holotrichia parallela* Larvae (Coleoptera: Scarabaeidae). *Int J Mol Sci.* 2012; **13**: 2563-2577.

Huang XM, Ge JL, Fan JX, Chen XL, Xu XH, Li JF, et al. Characterization and optimization of xylanase and endoglucanase production by *Trichoderma viride* HG 623 using response surface methodology (RSM). *Afr J Microbiol Res*. 2013; **7**: 4521-4532.

Jones D, Keddie RM. The Genus *Arthrobacter*. *Prokaryotes*. 2006; 3:945-960.

Kadarmoidheen M, Saranraj P, Stella D. Effects of cellulolytic fungi on the degradation of cellulosic agricultural wastes. *Int J Appl Microbiol Sci*. 2012; 1: 13-23.

Kageyama A, Morisaki K, Omura S, Takahashi Y. *Arthrobacter oryzae* sp. nov. and *Arthrobacter humicola* sp. nov. *Int J Syst Evol Microbiol*. 2008; 58: 53-56.

Kazemi A, Rasoul-Amini S, Shahbazi M, Safari A, Ghasemi Y. Isolation, identification, and media optimization of high-level cellulase production by *Bacillus* sp. BCCS A3, in a fermentation system using response surface methodology. *Prep Biochem Biotechnol*. 2014; 44: 107-118.

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, et al. Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol*. 2012; **62**: 716-721.

Kojima M, Okamoto K, Yanase H. Direct ethanol production from cellulosic materials by *Zymobacter palmae* carrying *Cellulomonas* endoglucanase and *Ruminococcus* β-glucosidase genes. *Appl Microbiol Biotechnol*. 2013; 97: 5137-5147.

Kumar S, Dudley J, Nei M, Tamura K. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform*. 2008; 9: 299-306.

Li XH, Yang HJ, Roy B, Park EY, Jiang LJ, Wang D, et al. Enhanced cellulase production of the *Trichoderma viride* mutated by microwave and ultraviolet. *Microbiol Res*. 2010, 165: 190-198.

Maki M, Leung KT, Qin W. The prospects of cellulose-producing bacteria for the bioconversion of lignocellulosic biomass. *Int J Biol Sci*. 2009; 5: 500-516.

Niewerth H, Schuldes J, Parschat K, Kiefer P, Vorholt JA, Daniel R, et al. Complete genome sequence and metabolic potential of the quinaldine-degrading bacterium *Arthrobacter* sp. Rue61a. BMC Genomics [Internet]. 2012 [cited 2015 Apr. 29]; 13, 534.

Pradeep MR, G Narasimha. Utilization of Pea Seed Husk as a Substrate for Cellulase Production by Mutant *Aspergillus niger*. *Insight Biotech*. 2011, 1: 17-22.

Shahzadi T, Mehmood S, Irshad M, Anwar Z, Afroz A, Zeeshan N, et al. Advances in lignocellulosic biotechnology: A brief review on lignocellulosic biomass and cellulases. *Adv Biosci Biotech*. 2014; 5: 246-251.

Sheng P, Huang SW, Wang Q, Wang AL, Zhang HY. Isolation, screening and optimization of the fermentation conditions of high cellulolytic bacteria from the hindgut of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). *Appl Biochem Biotechnol*. 2012; 167: 270-284.

Smibert RM, Krieg NR. Phenotypic characterization, In: Gerhardt P, Murray RGE, Wood WA, Krieg NR, Eds. methods for general and molecular bacteriology. Washington, D.C.: American society for microbiology Press; 1994. P. 607-654.

Soares FL, Melo IS, Dias AC, Andreote FD. Cellulolytic bacteria from soils in harsh environments. *World J Microbiol Biotechnol*. 2012; 28: 2195-2203.

Teather RM, Wood PJ. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol*. 1982; 43: 777-780.

Wang CM, Shyu CL, Ho SP, Chiou SH. Characterization of a novel thermophilic, cellulose-degrading bacterium *Paenibacillus* sp. strain B39. *Lett Appl Microbiol*. 2008; 47: 46-53.

Yuan SF, Wu TH, Lee HL, Hsieh HY, Lin WL, Yang B, et al. Biochemical characterization and structural analysis of a bifunctional cellulase/xylanase from *Clostridium thermocellum*. *J Biol Chem*. 2015; 290: 5739-5748.

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