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A NOVEL SCREENING METHOD OF CELLULASE-PRODUCING BACTERIA BASED ON *Phytophthora parasitica* var. *nicotianae*

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Cellulase is the key to utilize the renewable and abundant cellulose resource, cellulase-producing microorganism is an important source of cellulase. The traditional screening method of cellulase-producing microorganism is low efficacy and not macroscopic. The screening method in this study was based on the interactive culture character between cellulase-producing bacteria and *Phytophthora parasitica* var. *nicotianae* on plates, the results indicated that the inhibition zone and cellulase activity of bacterial strains are conformity on the whole, so the screening method is very quickly and apparent.

Cellulose biomass is a renewable and abundant resource with great potential for bioconversion to value-added bioproducts, the development of more efficient utilization of biomass has received increased attention in recent years [1–4]. However, the biorefining process remains economically unfeasible due to a lack of biocatalysts that can overcome cost hurdles, cellulases play an important role in processing biomass through advanced biotechnological approaches [5].

Cellulase-producing bacteria is a new source of cellulose [6, 7]. The traditional method is to analyze the enzyme activity after isolating a lot of bacteria. Screening for bacterial cellulase activity of microbial isolates was typically performed on carboxymethylcellulose (CMC) containing plates. This method can not be timely and zones of hydrolysis are not easily discernible. It is costed a lot of works, and the researcher can not directly know the results of enzyme activity [8].

Efficient plate-screening methods are a prerequisite which give a more rapid and highly discernible result [9–11].

An investigation of nine different species of *Phytophthora* has been made in order to determine the composition and structural pattern of the mycelium. A basic skeleton of chitin was found to have superimposed upon it a mixture of two forms of cellulose. The presence of a hexose was indicated, no pectic compounds were present. When viewed in polarized light, the same appearance is presented by young and old cultures of typical species of *Phytophthora* [12]. It was reported that the thickened and the cellulose-enriched hyphae cell wall of *Phytophthora* could be penetrated by *Pythium oligandrum*, which produced large amounts of cellulolytic enzymes [13].

This method was applied in the present study was that screening cellulase-producing bacteria was based on *P. parasitica* var. *nicotianae*. The cellulose of *P. parasitica* var. *nicotianae* was considered as objective substrate of cellulase from bacteria, and the results of experiments were direct and apparent.

MATERIALS AND METHODS

Experiment strains and mediums. *P. parasitica* var. *nicotianae* (wild type, Wang W. et al. [14]). Sesame medium [14]: sesame – 30 g, and sucrose – 15 g, deionized H_2O to 1000 ml, adjust the pH to 6.5. Screening medium (g/l deionized H_2O): sesame – 30, sucrose – 15, tryptone – 10, adjust the pH to 6.5. LB medium (Luria-Bertani medium) (g/l deionized H_2O): tryptone – 10, yeast extract – 5, NaCl – 10, adjust the pH to 7.0. Sterilize by autoclaving.

Isolation of candidate cellulase-producing bacteria. Soil samples were collected from the Chongqing University of Technology. Bacteria strains were isolated from soil samples with LB medium by traditional plate-isolating methods [15], which were cultivated at 28°C, the purified strains were conserved for further research.

Screening of cellulase-producing bacteria. The experiments were performed on the screening medium flat plates. The 0.4 cm diameter mycelial clump of *P. parasitica* var. *nicotianae* was inoculated on the center of the flat plate, and the candidate cellulase-producing bacteria were inoculated on both sides of *P. parasitica* var. *nicotianae*, the distance between them was 2 cm.They were cultured at 28°C for 3 days. The candidate bacteria with inhibition zones would be further examined their cellulase activities.

Assay of cellulase activity of the cellulase-producing bacteria. The culture fluid of candidate cellulase-producing bacteria were analyzed by dinitrosalicylic acid (DNS) and carboxymethylcellulose (CMC) containing plates standard method.

The strains were grown under aerobic conditions in LB at 28°C, followed by entrifugation at 4500 g for

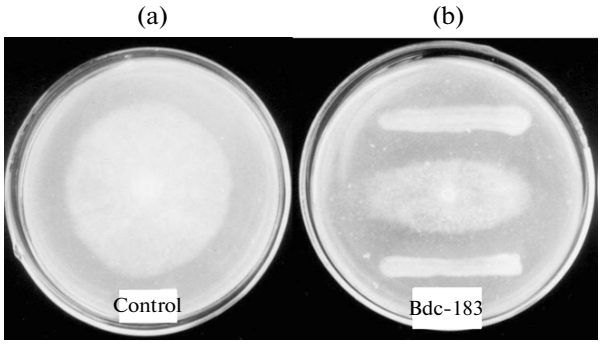

Fig. 1. Inhibition zone of bacterial strain Bdc-183; (a) control *P. parasitica var. nicotiana*; (b) *P. parasitica* var. *nicotiana* was on the center, Bdc-183 were on the both sides.

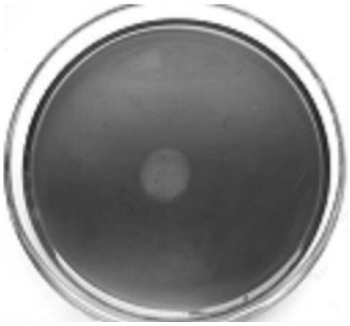

Fig. 2. The hydrolysis circle of bacterial strain Bdc-183 on CMC containing plate.

20 min, the supernatants were collected and adjusted to pH 5.3 with citric acid buffer for further experiments.

Supernatant was applied to analysis by DNS method [16]. 20 ml supernatant was dropped on the CMC containing plates, which were stained with Congo Red in 36 h [17–19].

Investigation of the hypha morphous of *P. parasitica* var. *nicotianae* by microscope. The sterilized glass slide was covered by screening medium for 1mm thickness, the *P. parasitica* var. *nicotianae* and Bdc-183 were inoculated on it, and the distance between them was 1.5 cm, they were cultured at 28°C for 48 h, and tested under microscope, the photomicrograph of anterior extremity hypha of *P. parasitica* var. *nicotianae* was taken.

RESULTS

Culture properties of strains on screening medium. 386 strains were isolated from soil samples, and 23 strains of which can inhibit the growth of *P. parasitica* var. *nicotianae*, for example, the inhibition zone between *P. parasitica* var. *nicotianae* and strain Bdc-183 was 10.3 mm, the inhibition zone between *P. parasitica* var. *nicotianae* and strain Bdc-53 was 3.5 mm (Fig. 1, table).

Analysis of cellulase activity of bacteria. The fermentation liquid of strain was analyzed by DNS method, and it indicated that the tested cellulase-producing bacteria with inhibition zones had different cellulase activities, and the cellulase activity of Bdc-183 was 13.50 U/ml, the cellulase activity of Bdc-53 was 5.43 U/ml.

The hydrolysis zones were very obvious on CMC containing plates, the diameter of hydrolysis circle of Bdc-183 was 0.98 cm (Fig. 2), the diameter of hydrolysis circle of Bdc-53 was 0.46 cm (results not shown). It were the same as DNS experiments.

The inhibition zone and cellulase activity of bacterial strains are conformity on the whole (table).

The hypha morphous of *P. parasitica* var. *nicotianae* hydrolyzed by Bdc-183. The anterior extremity hypha of *P. parasitica* var. *nicotiana* was hydrolyzed by bacterial strain Bdc-183, and the hypha was destructed into small pieces (Fig. 3, photomicrograph, 16 × 40). It was indicat-

Inhibition zone and cellulase activity of bacterial strains

Bacterial strains	Inhibition zone, mm	Cellulase activity, U/ml	Bacterial strains	Inhibition zone, mm	Cellulase activity, U/ml
Bdc-3	3.0	5.21	Bdc-183	10.3	13.50
Bdc-8	2.3	3.80	Bdc-189	4.0	5.68
Bdc-13	4.0	5.87	Bdc-197	2.5	4.42
Bdc-17	5.6	6.60	Bdc-231	1.5	2.41
Bdc-18	7.6	8.71	Bdc-243	2.8	4.86
Bdc-20	1.5	2.20	Bdc-308	3.8	5.36
Bdc-53	3.5	5.43	Bdc-345	8.5	9.27
Bdc-83	2.0	3.20	Bdc-367	1.8	3.22
Bdc-95	2.4	4.56	Bdc-369	4.5	5.96
Bdc-96	5.0	6.28	Bdc-372	5.0	6.68
Bdc-104	6.3	7.93	Bdc-381	1.0	1.98
Bdc-126	7.0	8.11			

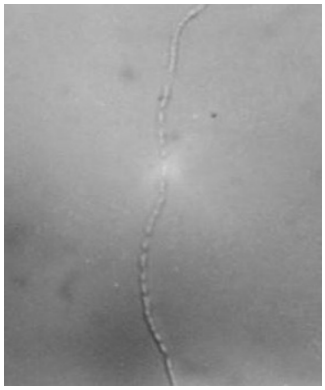

Fig. 3. The hypha of *P. parasitica* var. *nicotiana* was hydrolyzed by bacterial strain Bdc-183(photomicrograph, 16 × 40).

ed that the cellulase produced by Bdc-183 can decompose the hypha of *P. parasitica var. nicotiana* for its cellulose construction.

DISCUSSION

Efficient screening methods of cellulase-producing bacteria were constantly explored, which the course would be more rapid and the results would be highly discernible.

P. parasitica is a soilborne oomycete pathogen capable of infecting a wide range of plants, including many solanaceous plants. *P. parasitica var. nicotianae* is the pathogen of tobacco black shank [14], and it has the characteristic of *Phytophthora*,which is composed of hexose.

In this research, the composition characteristic of *P. parasitica var. nicotianae* was utilized, the *P. parasitica* var. *nicotianae* and cellulase-producing bacteria were cultivated correspondingly on screening medium plates, if the bacteria can produce cellulase, the *P. parasitica* var. *nicotiana* were restrained, the phenomenon can be easily detected, the screening efficiency will be greatly improved.

From the results described in the present study, it seems clear that the inhibition zone and cellulase activity of bacterial strains are conformity on the whole (see table). The inhibition zone and the cellulase of strain Bdc-53 were 3.5 mm and 5.43 U/ml, which of Bdc-183 were 10.3 mm and 13.50 U/ml, and which of Bdc-3 were 3.0 mm and 5.21 U/ml. It indicated that the results of the present study method and the classic method were conformity.

The mechanisms of antagonism between the bacteria and the fungus were investigated, the hypha of *P. parasitica* var. *nicotianae* was destructed by cellulase-producing bacteria for the cellulase.

Cellulose-produced bacteria inhibited the *P. parasitica* var. *nicotianae* on agar media, the aim bacteria can be easily detected by eyes, no need by instrument for further analysis. The method was rapid, simple and efficacy to identify cellulase-producing bacteria in a high throughput, and it is helpful to utilizing cellulose.

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