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**Research Article**

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# Screening and identification of a feruloyl esterase producing bacteria *Burkholderia fungorum* A216

**Anhao Jiao[1], Zhenshang Xu[1], Xiaoying Yang[1], Qin Guo[2] and Xinli Liu[1]***

[1]*Shandong Provincial Key Lab. of Microbial Engineering, Qi Lu University of Technology, Jinan, P.R. China*
[2]*Hualu Holdings Co., LTD. Jinan, P.R. China*

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**ABSTRACT**

*A bacterial strain named A216, with feruloyl esterase producing capability, was isolated from forest soil after enrichment. The strain was identified at the species level by morphology, physiological-biochemical tests and 16S rRNA gene sequencing assay. The results revealed that strain A216 was accurately identified as Burkholderia fungorum, and the ITS sequence of the species was submitted to the NCBI for the first time. The enzyme activity in different medium was preliminarily analyzed by spectrophotometric assay. After incubation 5 days, it gave the maximal activity of feruloyl esterase 14.24 U/L when cultivated in fermentation medium. However, no feruloyl esterase activity was detected in LB medium under the same condition. To our best knowledge, this is the first observation of feruloyl esterase activity in the species Burkholderia fungorum.*

**Keywords:** *Burkholderia fungorum*, feruloyl esterase, enzyme activity

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## INTRODUCTION

Feruloyl esterase(FAEs, E.C. 3.1.1.73), a subclass of carboxylesterases (E.C.3.1.1.1), are classified into four types(A, B, C, and D) based on their primary amino acid sequence identity and substrate specificity[1]. Feruloyl esterase in combination with other different activity enzymes degrade plant cell wall polymers completely[2,3], releasing ferulic acid and other cinnamic acid[4]. Contrast with chemical treatment, the use of feruloyl esterase to solubilize ester-linked phenolic compounds from a lignocellulose biomass would be more environmentally friendly. To this issue, there is a growing interest in feruloyl esterases as promising biocatalysts in the production of high value compounds from plant waste materials[5,6], as well as in the processing of hemicelluloses and pulp beaching[7,8]. In the future, feruloyl esterase will be needed in many biotechnological processes such as bio-refining, pharmaceutical and food industries [9,10,11].

Feruloyl esterase were first identified in *Streptomyces viridosporus* in 1987 [12]. To date, they have been characterized mainly in fungi[13] like *Aspergillus niger*[14] and *Aspergillus flavipes*[15], which were generally concerned by the researchers. Fewer studies have been performed on bacterial feruloyl esterases, with a few enzyme described in *Bacillus sp.*[16] or *Butyrivibrio sp.*[17]. In recent years new discoveries of feruloyl esterase producing strains include *Burkholderia multivorans*[18], *Aspergillus nidulans*[19], *Fusarium oxysporum*[20], *Aspergillus oryzae*[21] and *Dickeya dadantii*[22]. Although more and more feruloyl esterase producing microorganisms were detected, the yield of feruloyl esterase by these microbial fermentation is not meet the industrialized production requirements. It is necessary to screen new feruloyl esterase producing microorganisms.

In this paper we report the isolation, screening and identification of a *Burkholderia fungorum* isolate possessing feruloyl esterase activity using ethyl ferulate as a model substrate and the subsequent determination feruloyl esterase activity change by spectrophotometric method[23] relying on the measurement of 4-nitrophenol (4-NP) released

from 4-nitrophenyl ferulate (4-NPF) upon enzyme action. The study aims at enlarging a possible strain resources for production of feruloyl esterase.

## EXPERIMENTAL SECTION

### Collection of Sample

The soil sample was collected from the forest soil in Jinan, Shandong Province, China. It was collected within 10 min at ambient temperatures, kept on ice during transport and the enrichment was carried out immediately after the sample arrived at the laboratory.

### Bacterial Enrichment and Isolation

For the sample, approximately 3 g soil was added to 10 ml sterile water, concussion made into soil suspension. Then 0.2 ml of the suspension was added to an Erlenmeyer flask (100 ml) containing 20 ml enrichment medium, and incubated on a 200 rpm rotary shaker at 30℃ for 2 hours. The enrichment medium had the following composition (per liter): $(NH_4)_2SO_4$ 1.3g, $KH_2PO_4$ 0.37g, $MgSO_4{\cdot}7H_2O$ 0.25g, $CaCl_2{\cdot}2H_2O$ 0.07g, $FeCl_3$ 0.03g, yeast extract 1.0g, fungicidine 50000 U, with pH 6.5. After that, the 10-1 dilution was made by diluting 1 ml of the culture within 9 ml of physiological saline. Further tenfold serial dilution, ranging from 10-5 to 10-9 , were prepared and aseptically spread onto isolation medium plates which containing (per liter) NaCl 0.3g, $(NH_4)_2SO_4$ 1.3g, $MgSO_4{\cdot}7H_2O$ 0.3g, agar 15g and ethyl ferulate 15 ml (10％in dimethylformamide) as a single carbon source. Then the plates ware incubated aerobically at 30℃ for 3 days to obtain the feruloyl esterase producing strain. The selected colony was purified by repeated streaking on the isolation medium. Single colony of isolate was picked and streaked on fresh LB plate, for further study.

### 16S rRNA Gene and ITS Gene Sequence Identification

Total genomic DNA was extracted from 3 ml of overnight cultures at 30℃ by the CTAB method. The 16S rRNA gene was amplified by PCR using the universal primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The ITS gene were amplified using the following primers (5'-TGTACACACCGCCCGT-3') and (5'-CCTTTCCCTCACGGTACTG). They were amplified in automatic thermal cycler. Each sample contained 1×PCR buffer, 1.5 mM $MgCl_2$, 0.2 uM of each dNTP, 10pmol of each primer,10 ng template bacterial DNA and 1.0 U Taq DNA polymerase. In the negative control, the DNA volume was substituted by ultrapure water. The reaction conditions were 94℃ for 5 min, 94℃ for 30s, 54℃ for 30s, 72℃ for 90s, 30 cycles, and then 72℃ for 10 min. The amplified products were resolved by electrophoresis in 1.0％ agarose gels with stain agent Genegreen (Tiangen Biotech Co., Ltd, Beijing, China) and bands visualized with an UV imaging system. The PCR product of interest was isolated from agarose gel using a SanPrep Column DNA Gel Extraction Kit (Sangon Co., Ltd, Shanghai, China). The purified PCR products were then sequenced by Sangon Co.,Ltd.

### Sequence Analysis and 16S rRNA Gene Phylogenetic Tree

The sequencing result were analyzed and determined by basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST) and were submitted to the National Center for Biotechnology Information. Species identification was based on maximum score, identify and coverage values. Consensus sequences were imported into MEGA5.0 software (http://www.megasoftware.net/mega.php) to generate the phylogenetic tree using the neighbor-joining statistical method.

### Morphology and Physiological-biochemical Tests

Conventional identification was performed by using the following morphology and physiological-biochemical tests: light microscope observation; morphology in LB plate; gram staining; spore staining; MR test; VP test; indol production test; starch hydrolysis test; nitrate reduction test; gelatin liquefaction test; citrate utilization test; $H_2S$ production test; catalase production test.

### The assay of Enzyme Activity

Enzyme activity experiment was conducts according to the method of Mastihuba et al. 4-NPF was prepared according to the chemoenzymatic procedure described by Mastihubová et al[24]. Dissolved in DMSO and prepared the standard solution with different concentrations, the standard curve of 4NP and 4NPF were draw firstly. The slope of the standard curve represent the extinction coefficients of 4NP and 4NPF, respectively. Then enzyme activity was determined by measuring the production of 4NP from 4NPF. The substrate solution was prepared by mixing 8 vol of 0.1 M potassium phosphate buffer solution, pH 6.5, containing 2.5％Triton X-100 with 1 vol of 10 mM 4NPF in DMSO followed by immediate vortexing. The reaction mixture comprised 0.1 ml enzyme and 0.9 ml substrate solution. The absorbance around 0, 5, 10, 15 minutes were read at 410 nm in 10 mm path length quartz

cuvettes. In a control sample, the enzyme was replaced by the potassium phosphate buffer. Calibration curve were drew and the slope (k) of the regressing equation means the change of absorbance per minute. For practical reasons the reaction temperature was set up to ambient temperature (20℃). One unit of enzyme activity is defined as the amount of enzyme releasing 1 u mol of 4NP from 4NPF in 1 min under the assay condition. We can get corrected values for activity of per liter enzyme solution according to the equation:

$$\text{Enzyme activity (U/L)} = n \cdot \triangle A410 \cdot 106 / [(\varepsilon_p - \varepsilon_s) \cdot \zeta \cdot t], \quad (1)$$

where $\triangle A410 / t = k$, n is the dilution factor of the original enzyme solution when added to the reaction system, $\zeta$ is the path length, $\varepsilon_p$ is the extinction coefficient of the product 4NP, $\varepsilon_s$ is the extinction coefficient of the substrate 4NPF, and t represent the reaction time.

Bacterial strain was prepared by cultivation with 200rpm shaking in LB medium at 37℃ for 12 h. The starter culture (2%) was added to fermentation medium and LB medium to produce feruloyl esterase, and then cultivated on a 200rpm rotary shaker at 37℃. The fermentation medium had the following composition (per liter): $(NH_4)_2SO_4$ 1.3g, $KH_2PO_4$ 0.37g, $MgSO_4 \cdot 7H_2O$ 0.25g, $CaCl_2 \cdot 2H_2O$ 0.07g, $FeCl_3$ 0.03g, yeast extract 1g, wheat btan 20g with pH 6.5. Every 24 h the flasks of each sample were taken to collect supernatants by centrifugation and measure enzyme activity according to the above method. All experiments were carried out in triplicates and the presented results are average values of three independent experiments. The results were analyzed statistically and represented with a standard error.

## RESULTS AND DISCUSSION

### Isolation and Screening of the Feruloyl Esterase Producing Strain

A bacterial strain was isolated from the soil sample by the method mentioned above. The feruloyl esterase activity was confirmed by the transparent zone. The isolate, designated as A216, was found to possess the ability to produce feruloyl esterase, so it was chosen for further study.

### Identification of the Feruloyl Esterase Producing Strain

To confirm the species, the nucleotide sequence of the 16S rRNA gene and ITS gene of the strain were analyzed and determined by BLAST program at NCBI. The obtained 16S rRNA sequence and ITS sequence were deposited in GeneBank and assigned the following accession numbers: KJ026454 and KJ130021. Strain A216 was closely related to *Burkholderia fungorum* as it showed 99.78%homology to *B.fungorum* DBT1. Phylogenetic tree analysis was performed to show the relationship of 16S rRNA gene sequence between the isolate and related strains by using MEGA software (Fig. 1). While the ITS gene sequence showed a similarity of 97%to *Burkholderia phytofirmans* PsJN. The reason is possibly that in NCBI database there were not the ITS sequence information of the species *B.fongorum* before.

Light microscopy showed strain A216 was gram-negative, nonsporeforming and rod shaped, 0.4-0.5 um×0.9-1.5 um. The colony morphology in LB plate are small, circular, smooth, regular edge, opaque and milky. Combination between colony and medium is not closely. The physiological and biochemical characteristics listed in Table 1. Coenye et al. had a detail description of *B.fungorum*. Our results of indol production, nitrate reduction, gelatin liquefaction, citrate utilization are the common characteristics of *B.fungorum*. Catalase production is strain dependent and it is same with the type strain LMG16225T. MR test, VR test and $H_2S$ production test are not mentioned in that study.

**Table 1 Physiological and biochemical properties of strain A216**

| physiological-biochemical characteristics | result |
|---|---|
| MR test | - |
| VP test | + |
| Indol production test | - |
| Starch hydrolysis test | - |
| Nitrate reduction test | + |
| Gelatin liquefaction test | - |
| Citrate utilization test | + |
| $H_2S$ production test | - |
| Catalase production test | + |

*Characteristics are scored as: +, the strain tested gave a positive reaction; -, the strain tested gave a negative reaction.*

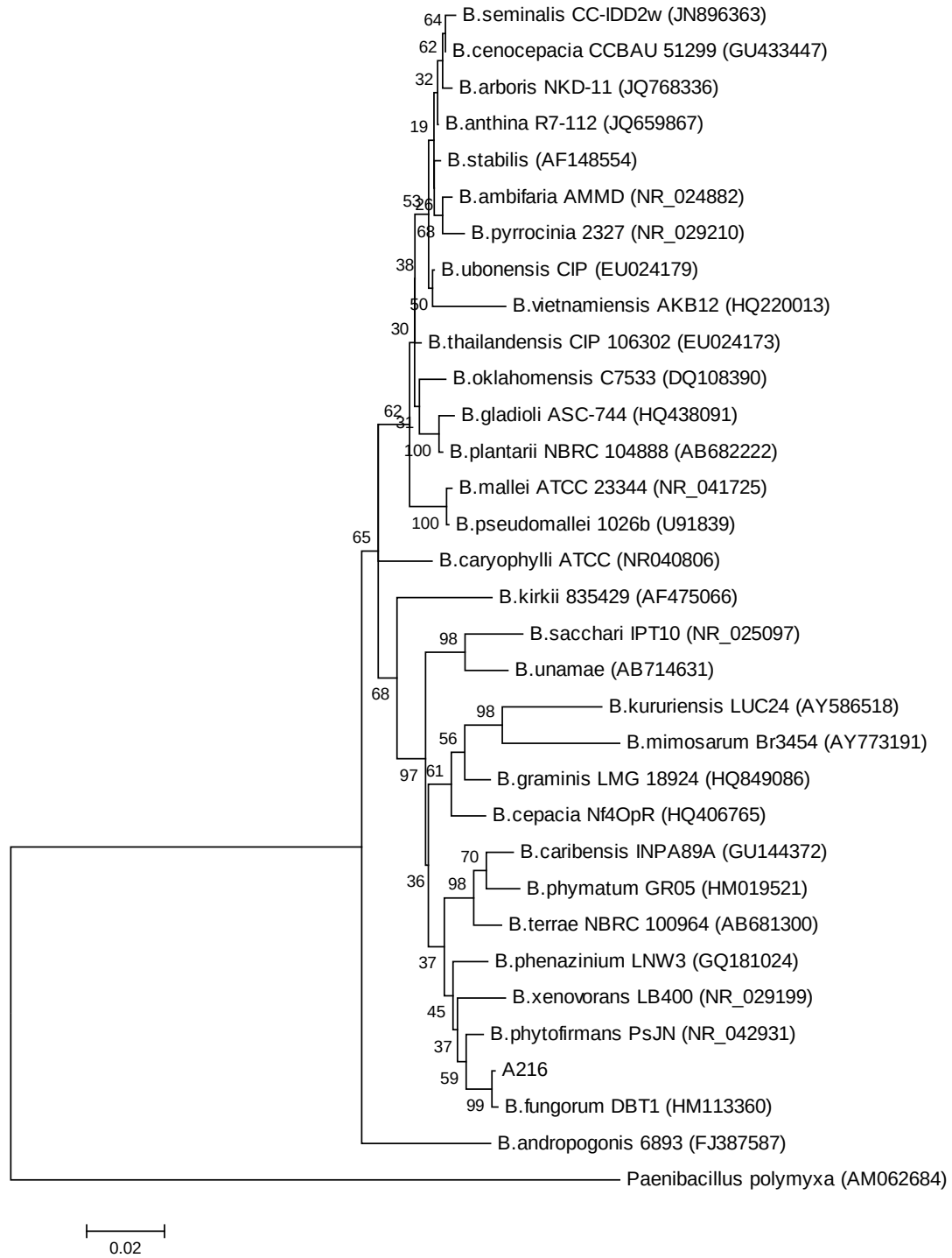


**Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences of strain A216 and related species. (The tree was evaluated by the neighbor-joining method based on 1000 replications. The scale bar represents 0.02 substitutions per nucleotide position, using *Paenibacillus polymyxa* (AM062684) as an outgroup)**

**Standard Curve for 4-NP and 4-NPF and Calculation of Enzyme Activity**

Under reaction conditions solutions of 4NP resulted in linear standard curves exhibiting correlation factors of 0.9996 and extinction coefficients of $2.0979\times10^3$ $dm^3mol^{-1}cm^{-1}$. The value of 4NPF were 0.9987 and $0.3069\times10^3$ $dm^3mol^{-1}cm^{-1}$, respectively(Fig. 2,3). The absorbance of ferulic acid was minimal and was neglected in the activity calculations (Mastihuba et al., 2002).

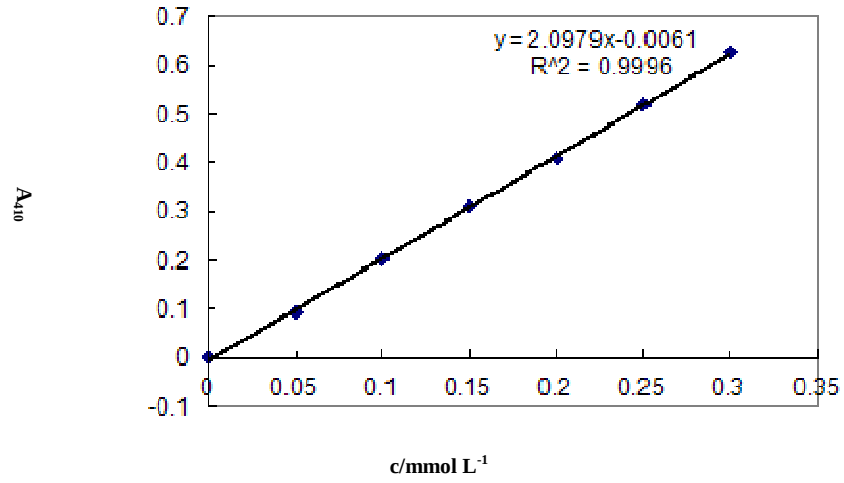


**Fig. 2 The standard curve of absorbance at 410 nm and 4-nitrophenol with different concentration**

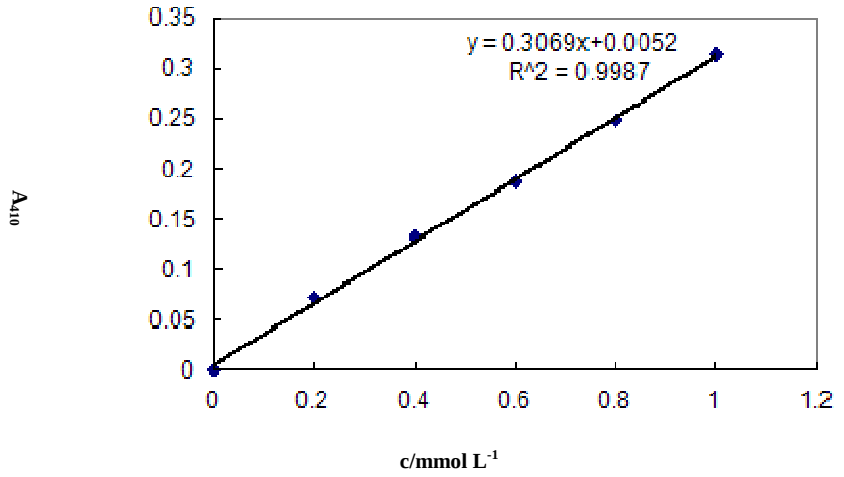


**Fig. 3 The standard curve of absorbance at 410 nm and 4-nitrophenol ferulate with different concentration**

Production of feruloyl esterase activity from *B.fungorum* grown on two different medium are shown in Fig. 4. Growth of *B.fungorum* on fermentation medium resulted in the production of feruloyl esterase activity which was characterized by culture supernatants. Esterase activity on 4-NPF was first detected 2 days after inoculation, increased evenly over days 2-5, obtained the maximal activity of 14.24 U/L at day 5, and then decreased gradually. We were not able to detect feruloyl esterase activity in cultures grown on LB medium.

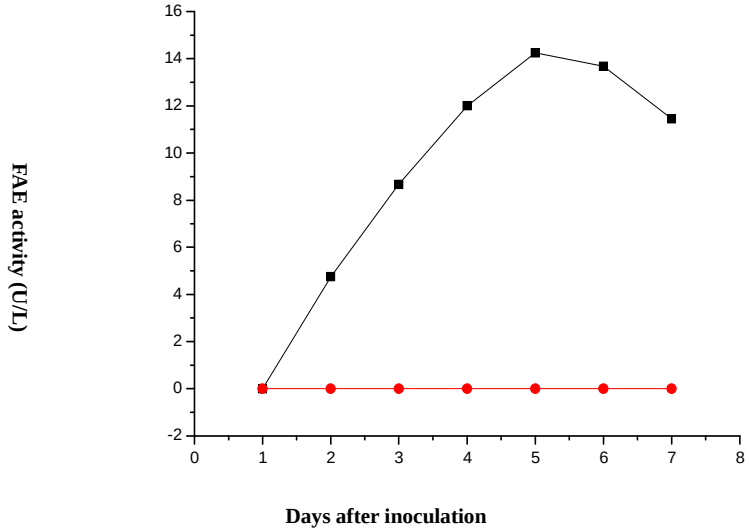


**Fig. 4 Production of feruloyl eaterase activity from *B.fungorum* grown on either fermentation medium or LB medium.(Enzyme activity was measured in culture supernants after growth on fermentation medium (■) or LB medium (●) using the spectrophotometric method with 4-NPF as substrate. The presented results are average values of three independent experiments)**

## DISCUSSION

It was reported that feruloyl esterase could act on not only natural substrates, such as xylan, pectin, wheat bran, and sugar beet, but also artificial substrates like ethyl ferulate and 4-nitrophenyl ferulate. When ethyl ferulate used as only carbon resource in the medium, the strains, which are able to produce feruloyl esterase, can utilize it and show a transparent area. By the size of transparent area around the colony, ferulyol esterase activity is preliminary determined. Using this method, feruloyl esterase producing strain A216 was isolated from the forest soil through enrichment, screening and purification.

Based on 16S rRNA gene sequence analysis and phenotypic characteristics, the isolate A216 was accurately assigned to the species *B.fungorum*. Coenye et al.[25] initially proposed the species *B.fungorum* for a group of 9 *B.cepacia*-like strains, which were isolated from the environment, animal and human clinical samples. In that study 4 strains were recovered from mouse and human clinical samples. There is no doubt that *B.fungorum* belongs to pathogenic microorganism. Another major source of *B.fungorum* are the white-rot fungus *Phanerochaete chrysosporium*, and it has been suggested there is a symbiotic relationship between them [26]. In the nature, *P.chrysosporium* can strongly degrade lignin and other wood components by releasing a series of enzymes, result in the wood rotted. So it is understandable that *B.fungorum* can produce feruloyl esterase which play a role in the degradation of plant cell wall.

Nowadays *B.fungorum* has been identified in a range of samples, but little is known about the species. To our best knowledge, this is the first observation of feruloyl esterase activity in the species *B.fungorum*. And we first submitted the ITS sequence of the species. Retrieved in the NCBI, a variety species of *Burkholderia* genus have feruloyl esterase gene, but few research and report were focus on this. In addition to Rashamuse KJ et al. obtained a novel recombinant feruloyl eaterase from *B.multivorans* genomic library.

Further tests were carried out to determine the enzyme activity change of strain A216. Most reported methods for measuring feruloyl esterase activity are based on HPLC techniques, using enzymatic hydrolysis of specific substrates such as hydroxycinnamic esters[27], plant polysaccharides[28] and so on. However, the disadvantages of these HPLC methods are expensive equipment-needed, time-consuming, and not suitable for rapid analysis of large numbers of sample. Here we used a spectrophotometric assay for the quantitative determination of feruloyl esterase. Based on the differences in spectral properties of 4-NP and its natural esters 4-NPF, it is accurate, rapid and easy to perform.

Two types of medium were prepared to determine the enzyme activity change. In fermentation medium, the wheat bran is a slowly available carbon source. Contrast with that, the nutrients of LB medium are easy to use. The result indicated that production of feruloyl esterase is regulated and inducible. The level of feruloyl esterase expression is tightly controlled by the available carbon source[29]. The gene is not expressed in the presence of readily utilizable

carbon source such as yeast extract or peptone. This is the reason why no feruloyl esterase activity was detected when cultivated in LB medium. While incubated in fermentation medium, the gene is expressed in the use of wheat bran after yeast extract is exhausted. It has been shown the presumed regulation mechanism, carbon catabolite repression, is involved in the regulation of a varied of genes[30].

For achieving its industrial applications, the chance to improve feruloyl esterase yield will depend on the successful discovery of novel microorganism which have high enzyme activity. This is the first report about *B.fungorum* had the ability to produce feruloyl esterase by utilizing inexpensive raw materials like wheat bran. It indicated that bacteria belonging to genera *Burkholderia sp.* may be a strain resource of feruloyl esterase production. This may broaden the current limited feruloyl esterase applications in the further.

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