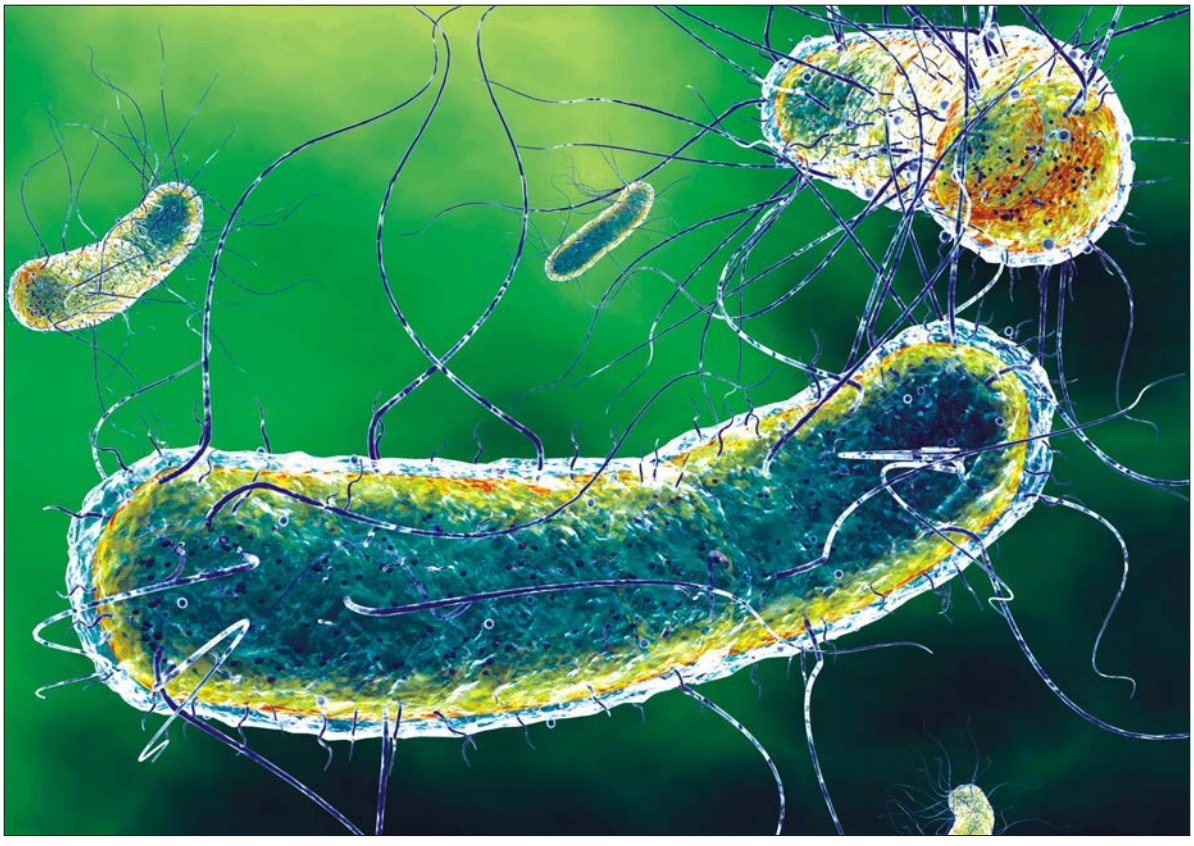

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# Isolation and Identification of *Bacillus altitudinis* ZJ 186 from Marine Soil Samples and its Antifungal Activity Against *Magnaporthe oryzae*

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## ABSTRACT

A strain *Bacillus altitudinis* ZJ 186 capable of producing antifungal compounds to inhibit the growth of seven tested physiological races of *Magnaporthe oryzae* was isolated from marine soil samples and characterized by biochemical and physiological identification as well as 16S rDNA sequence analysis. The filtrate from the culture broth of strain ZJ 186 showed strong growth inhibition against all tested physiological races of *Magnaporthe oryzae* indicating that suppression of the growth of the fungi was due to the presence of antifungal compounds in the ferment broth. Results showed that the filtrate exhibited antifungal activity at 20 h after inoculation and became significant correlated with the cell growth over 96 h cell growth period ($p<0.01$). Antifungal activity was relatively stable when the filtrate was exposed to conditions in the pH range 1-8, but was sensitive to alkalinity conditions. Moreover, the antifungal components lost partial activity when the temperature increased, but kept a quite thermally stable with approximately 40% of the activity of the culture filtrate being held at 60, 80 and 100°C for 30 min, respectively. It's the first report of bacterium *Bacillus altitudinis* showing antifungal capability against *Magnaporthe oryzae*.

**Key words:** *Bacillus altitudinis*, marine, isolation, antifungal activity, *Magnaporthe oryzae*

## INTRODUCTION

Rice blast caused by fungus *Magnaporthe oryzae* is one of the most serious rice diseases of the world and accounts for as much as 157 million tons yield losses from 1975 to 1990 (Baker *et al*., 1997).

Chemical control being as the main traditional measure is widely used in rice production to reduce the incidence of rice blast. But due to the development of resistance mutations and new physiological races of pathogens, chemical fungicides are gradually becoming ineffective (Zhao *et al*., 2010). At the same time, synthetic chemical fungicides can also cause environmental pollution because of their slow biodegradation (Zhang *et al*., 2011). Although hydrogen peroxide was reported to suppress the development of *M. oryzae*, it is difficult to popularize due to its side effects (Aver'yanov *et al*., 2007). It is becoming urgent to find new safer and more effective fungicides (Liu *et al*., 2001). The research in microbial secondary metabolites makes it possible in the search for such lead compounds (Liu *et al*., 2007).

The marine environment is the largest habitat on earth, accounting for more than 90% of the biosphere by volume, 71% by area and harbouring microorganisms responsible for about 50% of the total global primary production (Lauro *et al*., 2009). Natural product compounds from marine microorganisms are the source of numerous therapeutic agents (Vignesh *et al*., 2011). However, mainly because of their extreme living environments compared with terrestrial bacteria, the most majority of marine microbes have not yet been cultured (Alain and Querellou, 2009; Lang *et al*., 2005; Vartoukian *et al*., 2010). But it has become obvious that microorganisms from marine environments can also produce metabolites that are potential biocontrol agents (Chen *et al*., 2010). Secondary metabolisms produced by isolate from seaweed, seawater, sea cucumber and other marine samples were found to be with functions in antibacterial, antifungal, anti-inflammatory, antiviral and anticancer fields (Mokhlesi *et al*., 2012; Ramasamy and Kumar, 2009; Radjasa *et al*., 2007; Sugathan *et al*., 2012). As the development of biotechnology, more and more marine microorganisms be explored and their secondary metabolites provide a new strategy to plant fungi diseases control.

In this study, we isolated a marine bacterium, *Bacillus altitudinis* ZJ 186, which showed strong inhibition against the growth of seven tested physiological races of *M. oryzae*, including A1, B7, B15, B31, C9, D7 and G1. The strain was characterized by biochemical, physiological and 16S rDNA sequence analysis. Furthermore, the effects of pH and temperature on antifungal compounds and correlations between cell growth and antifungal activity of culture broth were investigated.

## MATERIALS AND METHODS

**Chemicals and reagents:** NaCl, $MgSO_4.7H_2O$, KCl, $CaCl_2$, $FePO_4$, $K_2HPO_4$, $FeSO_4$, $MnSO_4$, $CuSO_4$, glucose and L-glutamic acid sodium salt were all Analytical Reagent (AR) and purchased from Chang Zheng Glass and Reagents Co., Ltd., Sichuan. Potato Dextrose Agar (PDA) medium was purchased from Hangzhou Microbial Reagent Co. Ltd., China. Tryptone and Yeast Extract were purchased from Oxoid Ltd. PCR primers and Mixtures were provided by Beijing Genomics Institute. DNA Extract Kit and Purification Kit were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd.

**Isolation and characterization of strain ZJ 186:** The marine soil samples, collected from a near sea bottom of East China Sea, closed to Nan'ao island, Shantou, China, were serially diluted in sterile artificial sea water (NaCl: 23.4 g; $MgSO_4.7H_2O$, 24.6 g; KCl: 1.5 g, $CaCl_2$: 2.9 g, distilled water: 1000 mL) to $10^{-1}10^{-2}...10^{-6}$ and 100 μL aliquots of the different dilutions were plated onto Zobell 2216 medium (tryptone: 5 g; yeast extract: 1 g; $FePO_4$: 0.1 g; artificial sea water: 1000 mL; agar: 20 g; pH 7.6). Plates were incubated at 30°C for 12 h. Colonies representing different morphologies (color, texture, shape and size) were purified by restreaking on Zobell 2216 plates.

Strain ZJ 186 was identified by biochemical and physiological methods including cell staining, reactions with catalase and oxidase and fermentation assay (Vaneechoutte *et al*., 2000).

**16S rDNA analysis:** Total genomic DNA for PCR amplification of 16S rDNA was extracted from strain ZJ 186 following the method described in the literature (Sambrook *et al*., 1982). The 16S rDNA of strain ZJ 186 was amplified by PCR, using universal bacterial primers F27 (5'-AGAGTTTGATCATGGCTCAG-3') and R1492 (5'- TACGGTTACCTTGTTACGACTT-3') following Kim *et al*. (2007). The PCR products were purified and sequenced by Beijing Genomics Institute (Guangzhou, China). The Blast search was performed using similar 16S rDNA sequences data

which was deposited in NCBI GenBank (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA program version 4.1 (Kumar *et al*., 2004). The topology of the phylogenetic tree was evaluated by bootstrap resampling method of Felsenstein with 1000 replicates (Felsenstein, 1985).

**Test fungi:** Seven physiological races of *M. oryzae*, A1, B7, B15, B31, C9, D7 and G1, kindly provided by Molecular Genetic Lab of Rice Research Institute of Sichuan Agricultural University, were used as screening inhibitory indicators in the study. The representative physiological races A1 of *M. oryzae* was been chosen as tested fungi for activity assay.

**Antifungal activity assay:** Seed culture were prepared with a 150 mL Erlenmeyer flask containing 30 mL of Zobell 2216 broth medium and cultivated at 32°C for 12 h with shaking at 180 rpm. A 500 mL Erlenmeyer flasks was used for fermentation, containing 200 mL of Laddy medium (glucose: 20 g, L-glutamic acid sodium salt :5 g, $MgSO_4$: 0.5 g, KCl: 0.5 g, $K_2HPO_4$: 1 g, $FeSO_4$: 0.5 g, $MnSO_4$: 5 mg, $CuSO_4$: 0.16 mg, distilled water: 1000 mL) under conditions of an inoculation of seed culture volume of 2% (v/v), a temperature of 32°C, shaking at 180 rpm for 48 h. Free cells were removed by centrifugation at 6,000 rpm for 10 min and supernatant broth was sterilized by 0.22 μm filtration.

The inhibitory effects of culture filtrate on the growth of seven physiological races of *M. oryzae* were tested in Petri dishes (9 cm diam.) containing the potato dextrose agar (PDA) medium, following the published procedure (Gong *et al*., 2006) with minor modification. One milliliter of the culture filtrate mixed with 14 mL of the PDA medium was poured into a Petri dish. An inoculum (7 mm diam.) of each physiological race of *M. oryzae* was inoculated at the centre of PDA plate and held at 28°C for 7 d. Relative inhibition of fungal growth was evaluated by the percentage reduction in the growth of the mycelia in comparison to that on control plates without culture filtrate.

**Correlation between cell growth and antifungal activity:** A 1 L Erlenmeyer flask containing 400 mL of Laddy medium was inoculated with the seed culture (2%, v/v) of strain ZJ 186 and incubated at 32°C, 180 rpm for 108 h. During incubation, 2 mL of culture broth were taken at intervals for measurement of $OD_{600}$ and determination of antifungal activity against *M. oryzae* using the procedure described above.

**The effects of pH and temperature on the stability of antifungal filtrate:** The effects of pH and temperature on the stability of antifungal filtrate were assayed as previously described by Zhao *et al*. (2010) with slight modification. The culture filtrate of strain ZJ 186 was adjusted to various pH values in the range from 1.0 to 14.0 using 2 M HCl or 2 M NaOH and maintained at 4°C for 24 h. Antifungal activity was assayed after the samples were readjusted to pH 6.0. A similar procedure was used to conduct the effect of temperature on antifungal activity of the metabolites produced by strain ZJ 186. The culture filtrate was treated at temperatures of 32, 60, 80, 100 and 121°C, respectively, for 30 min and was tested for their antifungal activity to the representative physiological race A1 of *M. oryzae* after being cooled to room temperature.

**Statistical analysis:** All experiments were performed in triplicate and data are showed as mean values±standard deviation. A least significant difference (LSD) test with a confidence interval of 99% was used to compare the means.

## RESULTS

**Isolation of antifungal strain ZJ 186:** 589 strains were isolated from sea soil samples, of which 13 strains show distinct antifungal activity to *M. oryzae*. Strain ZJ 186 exhibited particularly strong activity and a broad spectrum of inhibition against all the seven physiological races of *M. oryzae* (Table 1).

**Identification of the strain ZJ 186:** Biochemical, physiological and metabolic characteristics of strain ZJ 186 was summarized in Table 2.

Bacterial isolate ZJ 186 is a rod shaped (1-1.2×2.5-4 μm), endospore forming gram positive bacterium. It is motile and capable of producing catalase and oxidase. The strain can use mannitol,

Table 1: Antagonism developed by 13 marine bacteria against seven physiological races of *M. oryzae*

| Strains | A1 | B9 | B15 | B31 | C9 | D7 | G1 |
|---|---|---|---|---|---|---|---|
| ZJ 126 | ++ | + | + | ++ | + | + | + |
| ZJ 182 | ++ | ++ | + | ++ | ++ | + | + |
| ZJ 186 | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| ZJ 220 | + | + | + | + | - | - | + |
| ZJ 311 | + | + | + | + | + | + | + |
| ZJ 315 | + | + | ++ | + | + | + | + |
| ZJ 336 | + | + | + | + | + | + | + |
| ZJ 337 | + | - | + | - | + | + | + |
| ZJ 382 | + | + | ++ | + | + | + | + |
| ZJ 387 | + | + | + | + | + | + | - |
| ZJ 392 | ++ | ++ | + | + | + | + | + |
| ZJ 419 | + | + | + | + | + | + | + |
| ZJ 435 | + | ++ | ++ | ++ | + | + | + |

-, +, ++ Represent relative inhibition of mycelium growth of each physiological races of *M. oryzae* on the PDA medium to the extent of ≤20, 20-50 and >50%, respectively

Table 2: Biochemical, physiological and metabolic characteristics of isolated strain ZJ 186

| Items | Results | Items | Results |
|---|---|---|---|
| Shape | rod | Sucrose | + |
| Size | 1–1.2×2.5-4 μm | Lactose | + |
| Endospore | + | Cellobiose | + |
| Gram stain | + | Raffinose | + |
| Motility | + | Melezitose | - |
| Catalase test | + | Dextrine | + |
| Oxidase test | + | Starch | + |
| Sorbitol | - | Esculin | + |
| Inositol | - | Urea | + |
| Mannitol | + | Salicin | + |
| Glucose | + | Citrate | + |
| Glucose aerosis | + | Glucose phosphate | - |
| Fructose | + | Acetate | + |
| Mannose | + | Nitrate reductase | - |
| Rhamnose | - | 6% NaCl | + |
| Xylose | + | 8% NaCl | + |
| Arabinose | - | 10% NaCl | + |

+: Positive; -: Negative

glucose, fructose, mannose, xylose, sucrose, lactose, cellobiose, raffinose, dextrine and starch as carbon sources, esculin, urea and salicin as nitrogen sources and showed NaCl tolerance of up to 10%. 16S rDNA gene sequence (1471 bp, Fig. 1) analysis (Accession No. AJ831842), followed by construction of phylogenetic tree by neighbour joining method, indicated that the strain ZJ 186 is *Bacillus altitudinis* (Fig. 2). Although *Bacillus* sp. along with *Streptomyces* sp. isolated from terrestrial samples were reported to exhibit antifungal activity to *M. oryzae* (Li *et al.*, 2011), it's the first report of bacterium *B. altitudinis* showing antifungal capability against *M. oryzae*.

**Correlation between cell growth and antifungal activity:** As shown in Fig. 3, the strain ZJ 186 grew slowly in Laddy culture broth. It needed 72 h to reach stationary phase. The filtrate exhibited antifungal activity at 20 h after inoculation and became significant correlated with the cell growth over 96 h cell growth period ($p<0.01$). The strongest activity was obtained at 72 h and maintained to 80 h, after which the activity declined slowly. Conclusion could be confirmed that under the culture conditions used in this study, the optimal time for the harvest of antifungal metabolites of the ZJ 186 is 72 to 80 h after inoculation.

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGAAGGGAGCTTGCTC
CCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGAT
AACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAA
AGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAA
CGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAA
GTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTA
GGGAAGAATAAGTGCAAGAGTAACTGCTTGCACCTTGACGGTACCTAACCAGAAAGCCAC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCG
GGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGT
GTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTC
TGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGT
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ACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGACAGAACAAAGGGCTGCGAGACCGCAAGGTTTAGCCAATCCCACAAA
TCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAA
TCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC
CACGAGAGTTTGCAACACCCGAAGTCGGTGAGGTAACCTTTATGGAGCCAGCCGCCGAAG

Fig. 1: 16S rDNA gene sequences (1471 bp) of the isolated strain ZJ 186

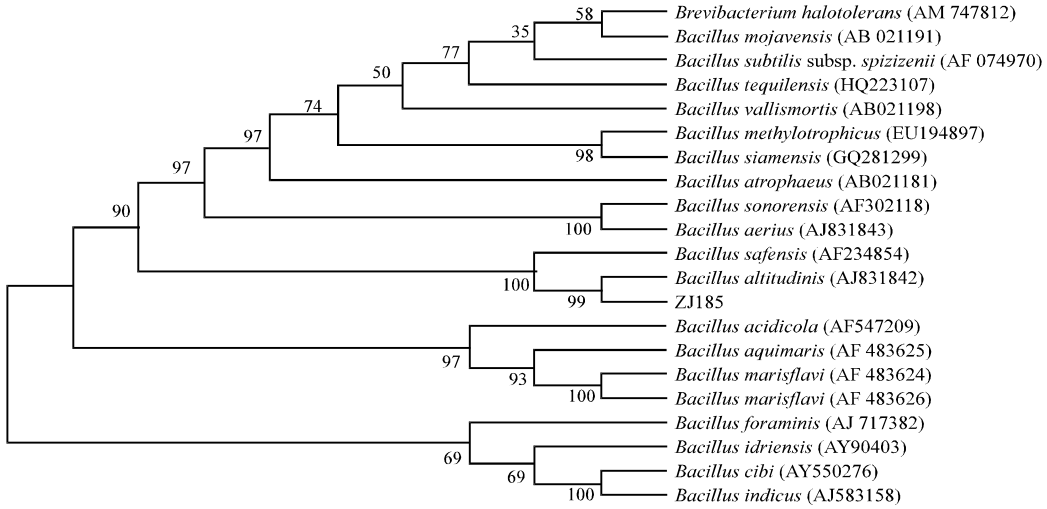


Fig. 2: Phylogenetic tree based on the 16S rDNA sequences of the showing affiliation of ZJ 186 strain with closely related members in GenBank. Phylogenetic trees were generated using MEGA version 4.1 with default parameters, K2P distance model and the Neighbor-Joining algorithm. The numbers at the branching prints are the percentages of occurrence in 1000 bootstrapped tree

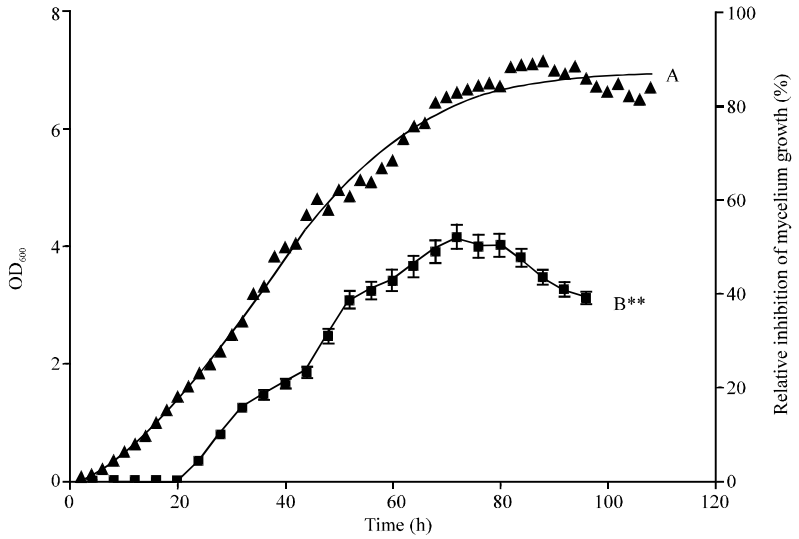


Fig. 3: Correlation between cell growth and antifungal activity. Line A: The growth curve of strain ZJ 186, Line B: The antifungal activities of the culture filtrate of the strain ZJ 186 against physiological race A1 of *M. oryzae*. **The correlation between the $OD_{600}$ of the strain ZJ 186 and the activity of its culture filtrate against tested fungi is significant at 1% level

**The effects of pH and temperature on the stability of antifungal filtrate:** The results shown in Fig. 4 demonstrated that the antifungal activity of the culture filtrate of the strain ZJ 186

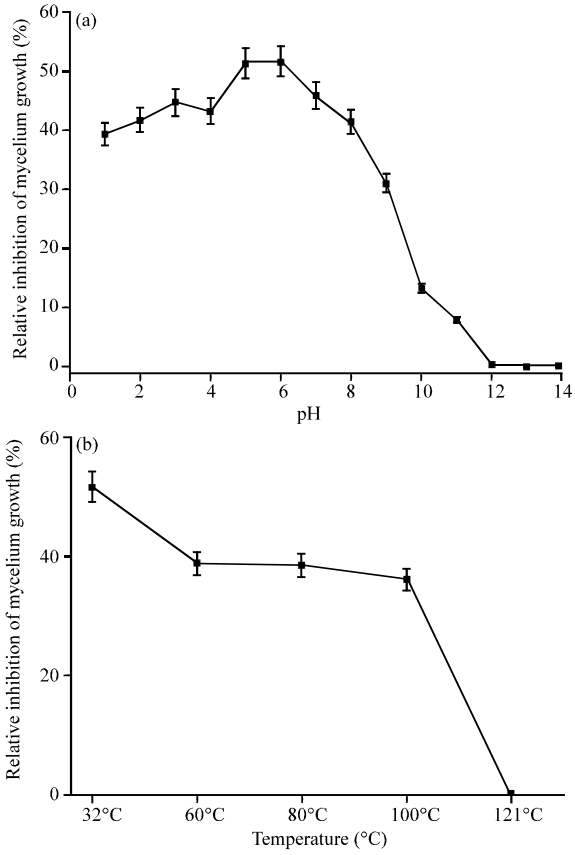


Fig. 4(a-b): Effects of pH and temperature on the antifungal activity of the strain ZJ 186 culture filtrate, (a) Samples of the culture filtrate were incubated at various pH values from 1.0 to 14.0 at 4°C for 24 h. Antifungal activity was assayed as the relative inhibition of mycelium growth of representative physiological race A1 of *M. oryzae* after the samples were readjusted to normal pH 6.0, (b) Samples of the culture filtrate were held at various temperatures for 30 min respectively. Antifungal activity was assayed after being cooled to room temperature. Data variance is indicated by bar

against representative physiological race A1 of *M. oryzae* maintained at a high level of 40-52% of relative inhibition of mycelium growth when the filtrate was exposed to conditions in the pH range 1-8 and reached to the highest level in the pH 5-6. However, activity reduced sharply after the filtrate being adjusted to alkalinity and disappeared completely when exposed to conditions of pH 12-14 (Fig. 4a). Moreover, the antifungal components lost partial activity when the temperature increased and disabled when it up to 121°C, but kept a quite thermally stable with approximately 40% of the activity of the culture filtrate being held at 60, 80 and 100°C for 30 min, respectively (Fig. 4b).

## DISCUSSION

Recently, antagonistic bacteria were used for biological control of plant pathogens infecting plant roots, leaves and sheaths (Wei *et al.*, 1991; Van Peer *et al.*, 1991; Maurhoffer *et al.*, 1994). As biocontrol agents, isolates of *Pseudomonas* and *Bacillus* have been the most studied and

exploited (Rahman *et al*., 2007; Kloepper *et al*., 1989; Glick, 1995; Ryder *et al*., 1994; Fuhrmann and Wollum, 1989). *Bacillus genus* was previously reported to show antifungal activity to *Ganoderma* pathogens, induce downy mildew resistance in Pearl Millet and cause diarrhea in goat (Rajendran *et al*., 2008; Chandrashekhara *et al*., 2007; Haque *et al*., 2007). The study of antagonistic bacteria and their antagonistic potential is important to understand their ecological role in diseases biocontrol and to elucidate their interaction with the host-plant (Jin *et al*., 2011).

Since *B. altitudinis* was firstly discovered to be a new species of *Bacillus* by Shivaji *et al*. (2006) from a air sample at a altitude of 41 km, few researches on this bacterium had been made, such as enhancing Jatropha oil extraction yield from the kernels assisted by *B. altitudinis* to preserve protein structure (Marasabessy *et al*., 2011), a novel serine alkaline protease was extracted from *B. altitudinis* GVC11 and it may be used as a dehairing agent (Kumar *et al*., 2011) and three types of carotenoids were found indicating a new carotenoid biosynthetic pathways existing in *B. altitudinis* (Khaneja *et al*., 2010). It was also reported to display antagonistic activity towards *Phytophthora nicotianae* (Jin *et al*., 2011). However, it's the first report of bacterium *B. altitudinis* showing strong antifungal capability against *M. oryzae*.

In view of the strain *B. altitudinis* ZJ 186 being isolated from marine samples, the culture conditions were extremely strict. From growth curve of strain ZJ 186 (Fig. 3), we could see it almost needed 72 h to reach stationary phase, several times of that of other bacterial (Liu *et al*., 2007). We tried several culture mediums and ferment conditions, only few of them could be used in antifungal activity assay (we summarized this content in another manuscript), which indicated the strain producing antifungal compounds only in optimal conditions.

The antifungal compounds were supposed to contain certain temperature-sensitive and thermo-stable substances, because the filtrate lost partial activity when it held in 60°C for 30 min, but kept almost unchanged in conditions of 60 to 100°C.

In conclusion, we isolated 13 strains from marine soil samples with different antifungal to the seven physiological races of *M. oryzae*. Strain ZJ 186 exhibited particularly strong activity and showed a broad spectrum of inhibition against all the tested fungus. Biochemical and physiological identification implied the strain belonged to *Bacillus* sp., which was further confirmed by 16S rDNA sequence analysis. We designated this strain to be *B. altitudinis* ZJ 186. A further study indicated that the culture filtrate had strong antifungal activity, which was significant correlated with the cell growth after inoculation 20 h. The antifungal compounds were relatively stable when the filtrate was exposed to conditions in the pH range 1-8, but was sensitive to alkalinity conditions. High temperature influenced slightly to the compounds until it rise up to 121°C for 30 min. It's the first report of bacterium *B. altitudinis* showing antifungal capability against *M. oryzae*. Although further studies should be conducted to purify active compounds and to elucidate the chemical formula, this study demonstrated that the culture filtrate of strain *B. altitudinis* ZJ 186 can be a potential antifungal substitution against rice blast caused by *M. oryzae*.

## ACKNOWLEDGMENT

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