

Production of a newly isolated *Paenibacillus polymyxa* biocontrol agent using monosodium glutamate wastewater and potato wastewater

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Received 12 October 2009; revised 13 November 2009; accepted 16 December 2009

Abstract

A phyllosphere bacterial strain EBL-06 was isolated from wheat leaves. The morphology, cultural characteristics, phospholipid fatty acids, physiological and antagonistic fungus activities of this strain were investigated. A phylogenetic tree was constructed by comparing with the published 16S rDNA sequences of the relevant bacteria. The results showed that the isolate EBL-06 was a strain of *Paenibacillus polymyxa*; this strain performed a high level of antagonistic fungus activity toward a broad spectrum of phytopathogens, such as *Botrytis cinerea*, *Cladosporium cucumerinum*, *Fusarium* spp. The isolate EBL-06 can grow well using monosodium glutamate wastewater (MGW) and potato wastewater (PW) as culture medium. The maximum yield of 6.5×10^9 CFU/mL of the isolate EBL-06 anti-fungus biocontrol agent was reached in 15 hr cultivation at 28°C, pH 6.0–7.5 using the mixture of MGW and PW (1:9).

Key words: phyllosphere; *Paenibacillus polymyxa*; wastewater; phospholipid fatty acids; biocontrol agent

DOI: 10.1016/S1001-0742(09)60267-9

Introduction

Effluents from monosodium glutamate manufacturing plants possess a high strength of COD (10,000–30,000 mg/L), ammonium (15,000–25,000 mg/L), sulphate (15,000–30,000 mg/L) and very low pH (< 2) (Yang et al., 2005). Effluents from potato chips industry possess a high load of starchy materials (Mishra et al., 2004). Because of the low pH, high concentration of COD, sulfate and NH₃-N, the treatment of such wastewaters by conventional activated sludge processes consume a lot of energy, resulting in high treatment costs (Bai et al., 2004).

Phyllosphere microbes often have a positive influence upon plant, where they may be involved in the fixation of nitrogen, promoting the growth of plants, or the control of plant pathogens. However, some phyllosphere microbes have negative effects upon the host where plant pathogens may result in disease (Zhang et al., 2009). The high infectivity fungi may damage many economically important crops and trees, and bring a significant risk and safety concerns for food supplies. Although chemical control agents have been used world widely, the biological control agents have attracted a great R&D interest because of their potential for long-term application as environmental friendly agents (ten Hoopen and Krauss, 2006).

Biocontrol agents have been used to protect plants against foliar diseases in several crops (Yuen and Schoneweis, 2007; Alvindia and Natsuaki, 2008; Perello et al., 2007). However, many of them are poor competitors for leaf surface nutrients compared with indigenous phyllosphere microbes (Zhang et al., 2008a; Lindow and Brandl, 2003; Lindow and Leveau, 2002).

Strains of *Paenibacillus polymyxa* have been isolated from different soils (Ash et al., 1993), rhizospheres and roots from plants cultivated all over the world, and many of them have been described as effective plant growth promoting rhizobacteria (Pichard et al., 1995; Petersen et al., 1996; Lorentz et al., 2006). However, *P. polymyxa* was seldom discovered in the phyllosphere before. In the present work, a strain of *P. polymyxa* EBL-06 was isolated from wheat phyllosphere, which could restrain the growth of the filamentous fungi. *P. polymyxa* strains have been proved to produce a wide variety of secondary metabolites, including different antibacterial and/or antifungal compounds (von der Weid et al., 2000). Therefore, the antagonistic effect of these strains upon microbial growth suggests a potential application as biological control agents. The aims of this study were to characterize the newly isolated strain of *P. polymyxa* EBL-06 and to optimize the production of the bacterium using potato wastewater (PW) and monosodium glutamate wastewater (MGW).

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1 Materials and methods

1.1 Isolation and *in vitro* antagonist assays

The experiment was conducted in a native field located at the Tongzhou near Beijing City, China. Wheat cultivars were planted in the field in October, 2006, watered and fertilized in accordance to local cultivation practices. Wheat phyllosphere microbes were collected in May, 2007 according to the methods described by Zhang et al. (2008b). An anti-fungi bacterial strain EBL-06 was isolated on Potato-dextrose Agar (PDA) medium Petri dish, and maintained on the PDA slant tubes. The antagonistic activity of the strain was evaluated by the method of confronting cultures with the filamentous fungi (Foldes et al., 2000; Bai et al., 2008), such as *Trichoderma harzium*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Fusarium* spp., *Macrophom* spp., and was modified as the following. A point of filamentous fungi was inoculated onto the center of PDA Petri dish; three points of isolation were inoculated at 2 cm distance from the center of the Petri dish symmetrically. The Petri dish was then incubated at 28°C for few days. The isolated strain was considered to be antagonistic to the filamentous fungus if it restrains the fungi growth with inhibition zone (Fig. 1).

1.2 Physiologic and resistance to antibiotics of bacteria

BIOLOG ECO microplates were used to analyze carbon source utilization patterns of the isolate EBL-06. The strain cultivated in the Luria-Bertani medium was collected by centrifugation and suspended with sterilized water to an OD of 0.12 at 600 nm, inoculated into the wells of Biolog ECO plate (150 µL/well) and incubated at 30°C (Holmes et al., 1994). Following 48 hr of incubation at 28°C, absorbance data were recorded at 590 nm with an ELISA Reader. The carbon was considered to be fully consumed if the OD₅₉₀ of the microwell was over 0.5. The soluble starch was also test as a carbon source by the medium (g/L): soluble starch 5.0, (NH₄)₂SO₄ 5.0, MgSO₄·7H₂O

0.1, KH₂PO₄ 0.5, NaCl 0.5, and K₂HPO₄ 1.5.

Resistance to antibiotics was determined using standard antibiotic disks. Inhibition diameters were recorded after 24 hr of incubation at 30°C under aerobic conditions. The classification of the strain, as sensitive, not sensitive or intermediate sensitive to the antibiotics, was done according to the inhibition diameters. Tests were performed in triplicates.

1.3 PCR amplification, sequencing, and phylogenetic analysis of 16S rRNA gene

The primer set 27F-1492R was used in PCR amplification of the 16S rRNA gene fragment of isolate EBL-06 under the conditions described by Kuklinsky et al. (2004). The 16S rDNA was sequenced by Shanghai Sangon Co. Ltd., China. Sequence similarity searches were conducted using the National Center for Biotechnology Information BLAST network service (nucleotide blast). Similar 16S rRNA gene sequences, from previously cultured bacteria, were downloaded from GenBank and manually checked for ambiguous sites using bioedit 7.0.1 software. Alignments were then performed against the 16S rRNA gene sequence of isolate EBL-06, where the pair-wise deletion option for gaps was employed. The alignment data were then used for neighbour-joining analysis with 1000 bootstrap replicates (MEGA version 4.0; Arizona State University, USA) (Li et al., 2007).

1.4 PLFA analysis

The EBL-06 was cultured in the Luria-Bertani medium overnight and was collected into a glass tube. The total lipid fractions were then extracted following the procedure as described by Bligh and Dyer (1959). Briefly, total lipid extracts were fractioned into neutral, glyco- and polar (phospho-) lipids by silicic acid chromatography. Phospholipids (the polar fraction) were converted to fatty acid methyl esters using a mild alkaline methanolysis reaction. An internal standard, fatty acid methyl ester (FAME) of

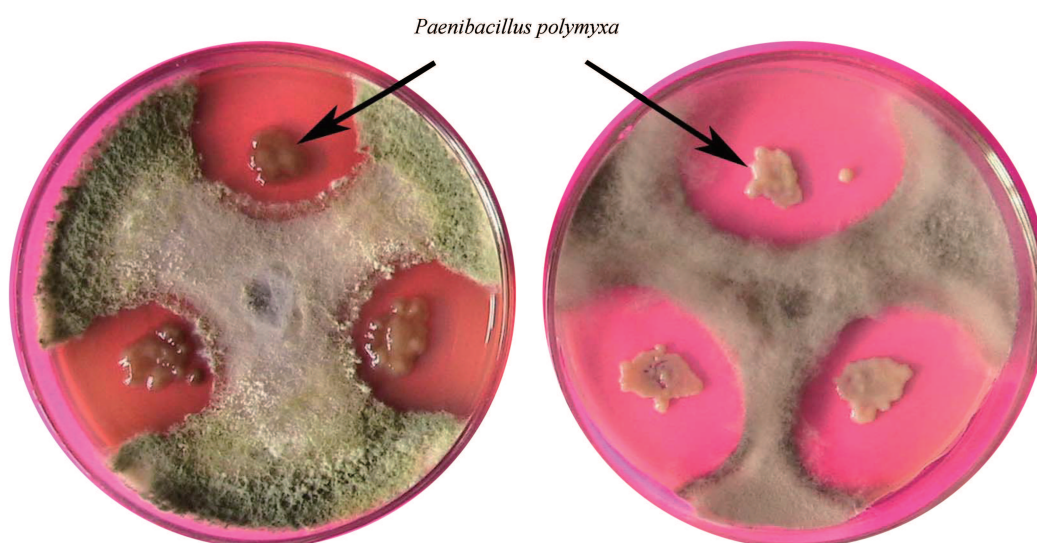


Fig. 1 Growth impact of the isolated EBL-06 on the *Trichoderma harzium* (left) and *Botrytis cinerea* (right) at the PDA medium.

C20:0 was added and dissolved in hexane for chromatographic analysis. FAMES were analyzed by a GC-MS system (Hewlett Packard HP 6890, Agilent Technologies Co. Ltd., USA) equipped with a HP-5 capillary column (30 m × 0.32 mm). For each sample, the abundance of individual FAMES was calculated in relation to the internal standard and the final result was expressed in terms of peak area.

1.5 Optimization of culture conditions

The PW was collected from a potato starch process waste stream, Beijing, China, mainly containing 15–30 g/L of potato starch, 20–40 g/L of total solids, 2.0–3.9 g/L of volatile suspended solids, 0.8–1.5 g/L reducing sugars and 0.5–0.9 g/L of total Kjeldahl-N. The pH of PW was 4.0; it was adjusted to 7.0 by NaOH when the PW was used as the culture medium. The starch in the PW was used as a main carbon source throughout the investigation. The MGW was obtained from Henan Lianhua Monosodium Glutamate Co., Ltd. (Zhoukou City, China), consisted of 1.20% total Kjeldahl-N, 1.17% NH_4^+ , 4.06% SO_4^{2-} and 0.275% reducing sugar. Trace element components in the MGW were given as follows (mg/L): Ba, 0.932; Ca, 389; Co, 0.0123; Cu, 0.605; K, 259; Mg, 79.0; Pb, 0.588; Sr, 0.869; Zn, 1.80; Mn, 2.66; Fe, 4.28; Na, 794; Cr, 0.862; P, 81.4; V, 0.0082. The pH of raw MGW was 1.5 and was adjusted to 7.0 by NaOH when the MGW was used as the culture medium. Ammonium in the MGW was used as the main nitrogen source.

The isolate EBL-06 was grown on the PDA slants at 30°C for 2 days. A single clone was inoculated into 250-mL flask containing 100 mL of PDA medium at 30°C for 12 hr. This seed culture was used to initiate the growth in fermentation medium used in this study. The basic medium (BM) is composed of as follows (g/L): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.10, KH_2PO_4 0.50, NaCl 0.50, and K_2HPO_4 1.50. The fermentation was conducted in 250 mL Erlenmeyer flasks containing 100 mL medium inoculated with 5 mL of seed culture. Unless otherwise was stated, the agitation rate and incubation temperature were 200 r/min and 30°C, respectively.

All experiments were conducted in duplicates and the average values are reported. Key results were repeated three times to establish their validity.

2 Results

2.1 Morphology and antagonistic fungus activity of the isolate EBL-06

Microscopic observation of the isolate EBL-06 is a mesophilic, Gram-positive and motile bacterium, and cells are rod-shaped with peritrichous flagella in overnight culture in PD medium at 30°C and 150 r/min. The cells are found singly, double, and chains. Colonies of the strain on PDA are slightly yellow, circular, smooth, convex, semi-transparent and 2–3 mm in diameter with an entire margin after incubation for 48 hr at 30°C.

EBL-06 showed a significant antagonistic activity to-

wards fungal species of *C. cucumerinum*, *T. harzimum*, *B. cinerea*, *Fusarium* spp., *Macrophom* spp. on PDA plates. Figure 1 shows two images of the test process.

2.2 Physiologic and resistance to antibiotics of the isolate EBL-06

Identified with BIOLOG-ECO plate, the isolate EBL-06 could use well the following carbon sources: α -cyclodextrin; glycogen; itaconic acid; D-galacturonic acid; 2-hydroxybenzoic acid; 4-hydroxybenzoic acid; L-arginine; L-asparagine; L-serine; glycyl-L-glutamic acid; D-xylose; D-mannitol; phenylethylamine. The other carbon sources in the plate can not be fully consumed in the test process. In addition, the isolate EBL-06 could grow well using soluble starch as carbon source.

The growth behaviour of the isolate EBL-06 was studied in the presence of a range of antibiotics. The strain was susceptible to penicillin, streptomycin, kanamycin and tetracycline, also was weakly susceptible to chloramphenicol, and resistant to polymyxine and colistine.

2.3 Phylogenetic analysis

To analyze the phylogenetic position, the 16S rDNA sequence of the isolate EBL-06 was determined, and a phylogenetic tree was constructed (Fig. 2). The sequence was deposited in the GenBank database under the accession number EF545556. The phylogenetic analysis indicated that the isolate EBL-06 is most closely related to species of *P. polymyxa*.

2.4 PLFA analyses

The individual PLFAs are given in Table 1. It is evident that the fatty acids 15:0, i15:0, a17:0 were the main PLFA.

2.5 Effect of carbon sources on the isolate EBL-06 growth

The fermentation medium contained 10 mL MGW, 90 mL BM, and 2% each carbon sources, including D-glucose, soluble starch, sucrose, molasses, and PW (90

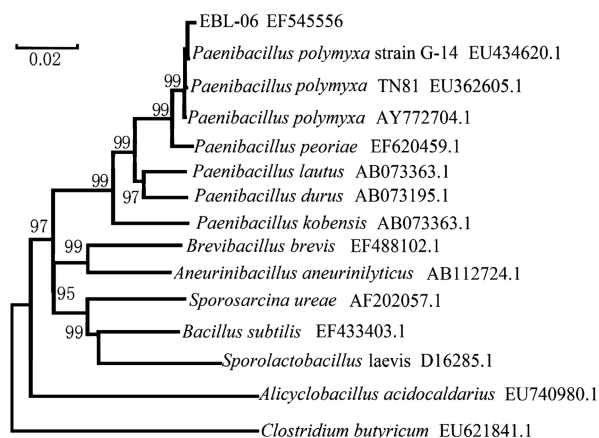


Fig. 2 Neighbor-joining trees showing the phylogenetic position of the isolate EBL-06 and its related species based on 16S rRNA gene sequences. The GenBank accession number for each microorganism used in the analysis is shown after the species name. Bootstrap values (expressed as a percentage of 1000 replications) greater than 50% are shown at the branches.

Table 1 Composition of the EBL-06 phospholipid fatty acids

Name	Molecular weight	Retention time (min)	Percentage (%)
14:0	242	15.14	0.71
15:0	256	16.75	13.36
i15:0	256	16.90	50.93
16:0	270	18.38	6.88
16:1 ω 9t	268	18.66	0.11
i16:0	270	18.98	4.09
17:0	284	19.99	4.55
a17:0	284	20.13	19.08
18:0	298	22.34	0.45
19:0	312	24.00	0.78

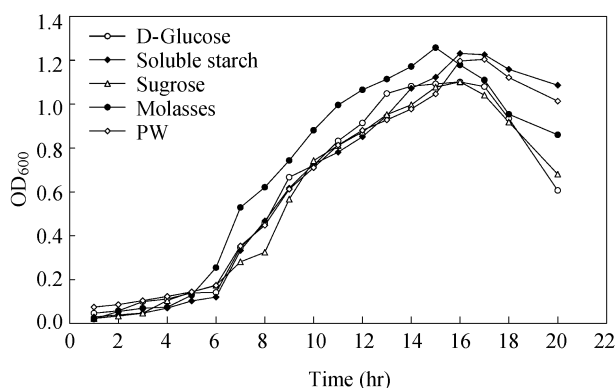
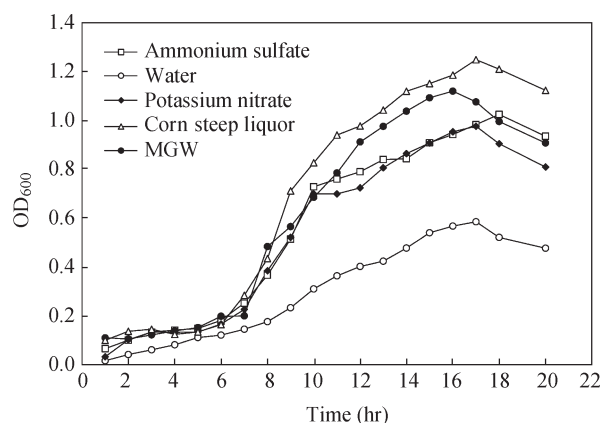
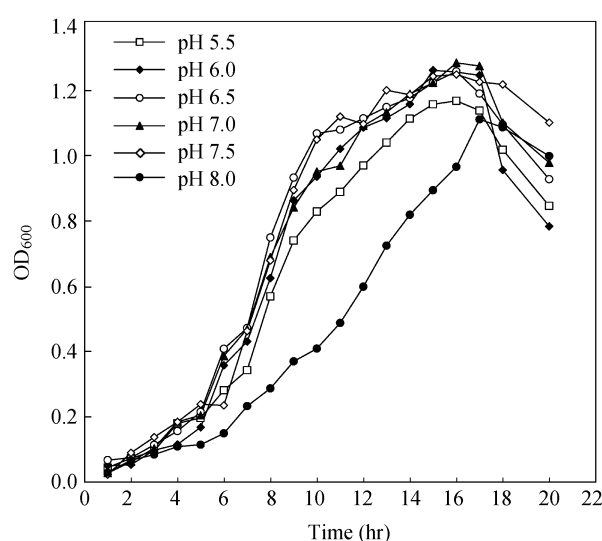
mL, the carbon source equal to 2 g starch, no BM). After inoculation with 2 mL inoculum, the medium was incubated at 30°C for 20 hr. The effect of carbon sources on the production of the isolate EBL-06 are presented in Fig. 3. It was found that molasses were the most efficient carbon sources for the production of strain. Comparing with the molasses, PW are the most suitable carbon source due to the low cost.

2.6 Effect of nitrogen sources on the isolate EBL-06 growth

The fermentation medium contained 90 mL PW as carbon source, 10 mL BM, and 0.5% different nitrogen sources, which were ammonium sulfate, water, potassium nitrate, corn steep liquor and MGW (10 mL, the nitrogen source equal to 0.5 g $(\text{NH}_4)_2\text{SO}_4$, no BM). A control experiment was conducted without addition of nitrogen source. After inoculation with 2 mL inoculum, the medium was incubated at 30°C for 20 hr. The results of impact of nitrogen sources on the production of isolate EBL-06 are presented in Fig. 4. It was found that corn steep liquor and MGW were the most efficient nitrogen sources for the production of strain. Comparing with the corn steep liquor, MGW are the most suitable nitrogen source due to the low cost.

2.7 Effect of pH on the isolate EBL-06 growth

The fermentation medium contained 10 mL MGW, 90 mL PW. After inoculation with 2 mL inoculum, the medium was incubated at pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 respectively. The isolate EBL-06 production at different

**Fig. 3** Effect of different carbon sources on the isolate EBL-06 growth. PW: potato wastewater.**Fig. 4** Effect of different nitrogen sources on the isolate EBL-06 growth. MGW: monosodium glutamate wastewater.**Fig. 5** Effect of initial culture medium pH on the isolate EBL-06 growth.

pH is shown in Fig. 5. The highest yield could be given at pH 6.0–7.5 after 15 hr fermentation. Consequently, pH 7.0 was selected in the following experiment.

2.8 Effect of culture temperature on the isolate EBL-06 production

The fermentation medium contained 10 mL MGW, 90 mL PW. After inoculation with 2 mL inoculum, the medium was incubated at 24, 28, 32, 36 and 40°C, respectively. The time courses of the isolate EBL-06 production at different temperatures are shown in Fig. 6. The maximum yield of 6.5×10^9 CFU/mL was reached at 28–32°C after 15 hr fermentation.

3 Discussion

P. polymyxa endospore was reported to be resistant to desiccation, heat, and UV irradiation, and have excellent biochemical characteristics that allow for further formulation and commercialization procedures. Previous studies have shown that strains of *P. polymyxa* can produce different peptide antimicrobial substances (Rosado

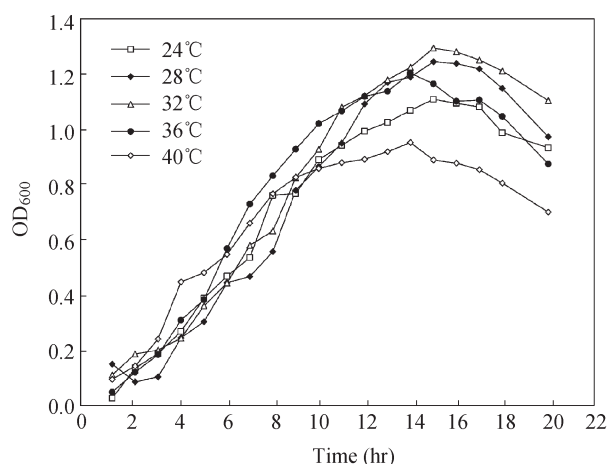


Fig. 6 Effect of culture temperature on the isolate EBL-06 growth.

and Seldin, 1993; Piuri et al., 1998; Dijksterhuis et al., 1999; Seldin et al., 1999). The peptide metabolites are generally classified into two groups according to their antimicrobial activities. The first group includes the polypeptides, polymyxins, jolipeptin, gavaserin, and saltavalin, which showed antibacterial activity against both Gram-negative and gram-positive bacteria. The second group consists of a single family of closely related peptides variously designated gatavalin, fusaricidins, all of which contain an unusual fatty acid side chain, 15-guanidino-3-hydroxypentadecanoic acid (Raza et al., 2009). As a phyllosphere isolate, *P. polymyxa* EBL-06 could be suitable to survive in the phyllosphere conditions; the antagonistic fungi ability also give them a growth advantage in the competitive environment. *P. polymyxa* EBL-06 showed a significant antagonistic activity towards all the filamentous fungi tested. It will be used as a potential biocontrol agent for protecting plant against fungal disease in further study.

MGW is one of the most intractable wastewater because of its high strength of COD, ammonium and sulphate (Yang et al., 2005). PW is also a high concentration of COD wastewater. A few studies have focused on the utilization of such high concentration of organic matter wastewater (Bai et al., 2004; Mishra et al., 2004; Huang et al., 2005). As far as the authors are aware, no article has been published on utilization of both MGW and PW in one process. In the present study, the isolate EBL-06 could use well the starch in the PW as the main carbon source, and ammonium in the MGW as the main nitrogen source. In addition, the nutrient and trace element components in the wastewaters are also suitable for the isolate EBL-06 growth. Compared with other culture media, the mixture of PW and MGW is the most suitable for the biocontrol agent production from the economic and environmental point of view.

P. polymyxa has also been isolated from several places such as food, rhizosphere, poultry production environments, and soils; and most of them can restrain fungal pathogen growth (He et al., 2007; Raza et al., 2009; Svetoch et al., 2005). Further studies will investigate the anti-fungal pathogen activity of the isolate EBL-06 both

in phyllosphere and in rhizosphere by field trials. Further understanding of the survival strategy of the isolate EBL-06 in phyllosphere might improve the efficiency of the biological treatments and also lead to enhanced yields of agricultural crops.

4 Conclusions

A newly isolated *P. polymyxa* EBL-06 from wheat phyllosphere can be used as a nonchemical alternative biocontrol agent against plant disease caused by fungal pathogen. A novel process for economical production of *P. polymyxa* biocontrol agent was developed using MGW and PW. It is feasible to develop a hybrid biotechnological process, integrating the production of environmental friendly biocontrol agent with the treatment of intractable wastewater.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 30600082), the Knowledge Innovation Program of Chinese Academy of Sciences (No. KSCX2-YW-G-054-2) and the Key Technologies R&D Program of China (No. 2008BADA7B01). We are grateful to Mr. Lidong Gao and Yang Zhou from Henan Lianhua Monosodium Glutamate Co., Ltd., for their help in MGW supplying.

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