Journal of Biofertilizers & Biopesticides
ISSN: 2155-6202
Hongzhang, et al. J Biofertil Biopestici 2012, 3:1
DOI: 10.4172/2155-6202.1000112
Research Article Open Access

Production of Protein Feed from Sweet Sorghum Stalk by the Two-Step Solid State Fermentation

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Abstract

Ensiling of agricultural straw produces feed with low protein content. To get high protein feed with high production and low cost, a two-step solid state fermentation was designed and several factors were optimized to maximize the yield. *Candida tropicalis* was first inoculated sweet sorghum stalk under aerobic condition for cell growth and protein content (PC) in the substrate after the first-step reached 26.3%. In the second-step, *Lactobacillus rhamnosus* was inoculated the sterilized substrate harvested from the first step, and cultured under anaerobic condition. After *L. rhamnosus* fermented at 40ºC for 80 h, the protein content in the product reached 35.7%. The pilot-plant scale fermentation was carried out according to the optimized conditions. By the two-step fermentation, 200 tons of feed was produced from two tons of dry sweet sorghum stalk and the protein content in the product was 13.77%, higher than silage in sale. The two-step process, which was easy to operate, provided high protein feed from agricultural waste in a cost effective way and the optimization of fermentation conditions in the two-step fermentation provided theoretical guideline for plant-scale production.

Keywords: Protein feed; Sweet sorghum stalk; Two-step solid state fermentation; *C. Tropicalis; L. rhamnosus*

Introduction

Sweet sorghum, because of its high yields and high sugar content, has attracted great attention as feedstuff and energy crop [1]. Of all usages, sweet sorghum stalks are mainly ensiled [2,3] or directly used as animal feed. Ensiling is a process of solid state fermentation by mixed bacteria of nature, however, the silage obtained has a low protein content (4-8%) with poor palatability. Great attempts on microbial fermentation have been made to enhance protein content in agricultural straw for poultry feed [4,5,6,7], but there is no report about large scale production of high protein feed in markets.

Solid state fermentation has the advantage of energy-saving and pollution-reducing [8,9], and it is usually applied in feed fermentation as its product can be utilized directly as feed. Therefore, solid state fermentation was chosen for conversion of sweet sorghum stalk to protein feed.

C. Tropicalis is widely used in production of single cell protein [10] because it's rich in protein and all essential amino acids and also because of its high digestibility. *L. rhamnosus* was generally used for lactic acid production [11] which could improve feed quality and inhibit the growth of undesirable microorganisms. Therefore, *C. Tropicalis* and *L. rhamnosus* were chosen as microbes for fermentation. Sugar content in sweet sorghum stalk is 50% or so, which is excellent carbohydrate for *C. Tropicalis*. However, *Lactobacillus* has a harsh demand on the nutrition of medium. A variety of amino acids and growth factors are requisite in the fermentation medium [12]. The yeast extract is stated to be an excellent source of nitrogen [13,14,15] for *Lactobacillus* in almost all studies of nitrogen compound. However, due to high cost of yeast extract, the application of *Lactobacillus* in feedstuff fermentation industry has been restricted. In this study, low-cost, excellent source of organic nitrogen was provided for *Lactobacillus* by the first-step fermentation.

In this paper, sweet sorghum stalks was used as substrate, and a two-step solid state fermentation process was developed to convert sugar in the substrate into protein for production of high protein feed.

Materials and Methods

Materials

Sweet sorghum was harvested in September at Inner Mongolian Autonomous Region, China. Sugar contents in the fresh stalk were (g/100 g sweet sorghum stalk): glucose, 1.8; fructose, 2.1; sucrose, 15.9; cellulose, 6.7 and the initial moisture content was 72±1.51%. The panicle and root were removed before the stalks were chopped into 0.5-1.0cm.

Microorganisms and culture media

C. Tropicalis CGMCC 2.587 (obtained from Institute of Microbiology, Chinese Academy of Sciences) and *L. rhamnosus* CICC 6003(obtained from China Center of Industrial Culture Collection) were stored at 4ºC and subcultured every 2 months. *C. Tropicalis* was maintained on potato sucrose agar slants (g L^{-1}): potatoes, 200; sucrose, 10. *L. rhamnosus* was maintained on De Man, Rogosa and Sharpe (MRS) slants (g L^{-1}): glucose, 20; peptone, 10; beef extract, 10; yeast powder, 5; sodium acetate, 5; ammonium citrate, 2; MgSO4•7H2O, 0.1; KH_2PO_4, 2; $MnSO_4•H_2O$, 0.05; Tween 80, 1.

Seed culture for the first and second-step fermentation

The seed culture medium for *C. Tropicalis* contained 20 g glucose, 10 g yeast extract, 20 g peptone per liter water and seed culture medium for *L. rhamnosus* was (g L^{-1}): glucose, 20; peptone, 10; beef extract, 10;

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Received November 21, 2011; **Accepted** December 19, 2011; **Published** December 25, 2011

Citation: Hongzhang C, Yumei W, Shuhua D (2012) Production of Protein Feed from Sweet Sorghum Stalk by the Two-Step Solid State Fermentation. J Biofertil Biopestici 3:112. doi:10.4172/2155-6202.1000112

yeast powder, pH 6.5. All the media were autoclaved at 115°C for 20 min before use.

Seed culture of *C. Tropicalis* was grown in a 250 ml flask containing 100 ml medium and incubated at 28°C on a rotary shaker at 180 rpm for 20 h, when the cell density reached 240 million mL^{-1}.

Seed culture of *L. rhamnosus* was grown in a 250 ml flask containing 100 ml medium and incubated at 40°C on a rotary shaker at 180 rpm for 14 h, when the optical density of OD_{600} was 1.1.

The two-step solid state fermentation

Solid state fermentation was carried out in 250 ml flasks, each of which contained 100 g chopped sweet sorghum stalks. First, *C. Tropicalis* was inoculated the substrate and cultivated under aerobic condition; then the whole substrate was autoclaved at 115°C for 20 min and cooled to room temperature. In the second step, *L. rhamnosus* was inoculated the sterilized substrate and cultivated to get high cell protein.

The effects of addition quantity of $(NH_4)_2SO_4$ (0.5, 1.0, 1.5, 2.0, 2.5 g water/g dry sweet sorghum stalk) on *C. Tropicalis* fermentation was investigated. The initial moisture content(g water/g dry sweet sorghum stalk) of the substrate (55±1.53, 60±1.39, 65±1.31, 70±1.22, 75±1.45%), inoculation amount (3, 4, 5, 6, 7, 8, 9, 10, 11, 12%), pH (3, 4, 5, 6, 7), temperature (28, 30, 32, 34, 36, 38, 40, 42, 44°C) and incubation period (18, 20, 22, 24, 26, 74, 76, 78, 80, 82, 84 h) were investigated separately in the two-step fermentation. The optimum condition of each single factor achieved in each optimization step was fixed for subsequent experiments.

Analytical methods

The moisture of sorghum stalk, neutral detergent fiber and acid detergent fiber content were determined by the Van Soest method [16]. Residual sugar was determined using the 3,5-dinitrosalicylic acid (DNS) method [17,18]. Water soluble carbohydrate was determined by sulfur- method [19].

Protein content in the substrate was determined by a Kjeldahl technique [20]: The dry sample of 0.5 g was added into a 500ml Kjeldahl flask containing 20ml of concentrated sulfuric acid, $CuSO_4$ 0.6g and K_2SO_4 2.4g. The sample was turned into blue liquid by being heated on electric furnace for 2-3h. Then 10 ml of liquid sample and 10mol L^{-1} NaOH were added into Kjeldahl instrument. The distillation process was achieved in the Kjeldahl instrument. The ammonium liberated was collected by distillation and recovered in boric acid solution. Subsequent titration with hydrochloric acid was done to calculate the initial amount of ammonium present in sample.

The amount of acetic acid, lactic acid and butanoic acid in the fermented product was quantified by a HPLC system (Agilent technology 1200 series, Palo Alto, CA, USA), using a Aminex HPX-87H ion exclusion column (Bio-Rad, Sunnyvale, CA, USA). The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}. For HPLC analysis, samples were filtered through a 0.45 lm filter and diluted properly with H_2SO_4 (5 mM).

Results

Effect of addition quantity of $(NH_4)2SO_4$ on *C. tropicalis* fermentation

The effect of addition quantity of $(NH_4)2SO_4$ on *C. Tropicalis* fermentation was evaluated. Fermentation was carried out with inoculation amount 5% and natural pH at 32°C for 20h. As is shown in Figure1, with the increase of added ammonium sulfate, protein content increased correspondingly. But residual sugar content decreased and was lowest when adding 1.5% (g/g dry sweet sorghum stalk) ammonia sulfate, beyond which the residual sugar content was higher.

Effect of moisture levels of the substrate on protein content in the product

Different moisture levels of the substrate in the two-step fermentation were carried out with inoculation amount 5% and natural pH at 30°C for 20h in the first-step and inoculation amount 10% and initial pH 6.5 at 40°C for 80h in the second-step. The moisture content of the substrate was adjusted before autoclaving. The results were shown in Figure 2. The highest PC was obtained at initial moisture level of 65±1.31% in the first-step and 70±1.22% in the second.

Effect of inoculation amount on protein content in the product

With initial moisture level of 65±1.31% and natural pH at 30°C for 20h in the first-step and initial moisture level of 70±1.22% and initial pH 6.5 at 40°C for 80h in the second-step, the effect of different

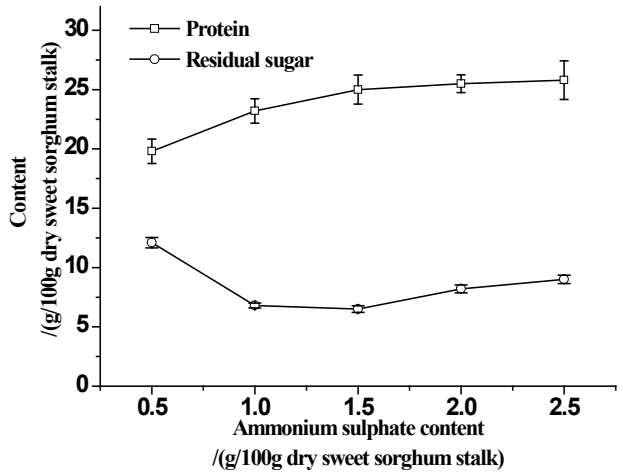

Figure 1: Effect of addition quantity of $(NH_4)2SO_4$ on *C. tropicalis* fermentation.

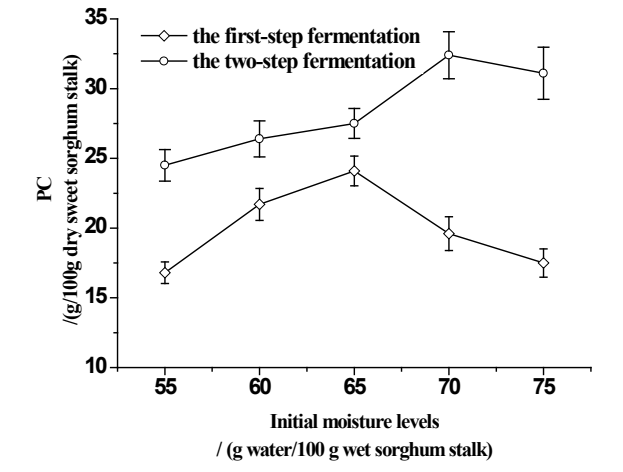

Figure 2: Effect of initial moisture levels on each step fermentation.

inoculation amounts on the two step fermentation was evaluated. Figure 3 indicated that with the increase of inoculation amount, PC was also increasing, but beyond the point of 6% in the first-step and 10% in the second-step it stabilized. Therefore, inoculation amount of 6% in the first-step and 10% in the second-step were chosen for high protein production.

Effect of initial pH of the substrate on protein content in the product

In the second-step fermentation, *L. rhamnosus* was incubated with initial moisture level of 70±1.22% and inoculation amount of 10% at 40°C for 80h. Figure 4 showed changes of PC with pH varying from 3 to 7 in the second fermentation step. PC reached the highest at pH 6.0 beyond which it declined, and meanwhile, sugar in the substrate was also completely consumed. Therefore, pH was chosen as 6.0 for the second step fermentation.

Effect of incubation temperature on protein content in the product

To study the effect of incubation temperature, fermentation was carried out at 28, 30, 32, 34, 36°C with initial moisture level of 65%, natural pH and inoculation amount 6% for 20h in the first-step, and 36, 38, 40, 42, 44°C with initial moisture level of 70±1.22%, initial pH 6 and inoculation amount 10% for 80h in the second step. Figure 5 indicated the suitable temperatures for growth of *C. Tropicalis* and *L. rhamnosus* were 32°C and 40°C, respectively. At temperatures lower or higher than the optimum, less PC was observed.

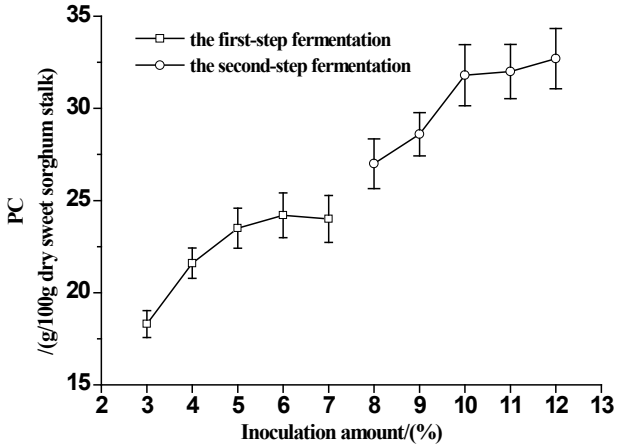

Figure 3: Effect of inoculation amount on each step fermentation.

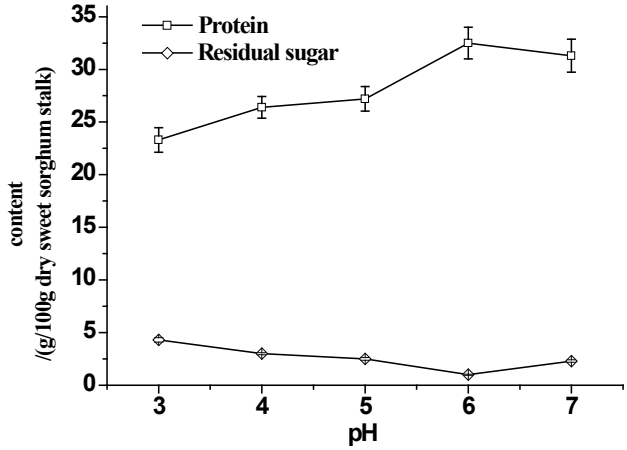

Figure 4: Effect of pH on *L. rhamnosus* fermentation.

Determination of fermentation time

Different incubation periods in the first and second step were employed to determine an optimal time for high PC. And inoculation time for the second-step fermentation was also analyzed.

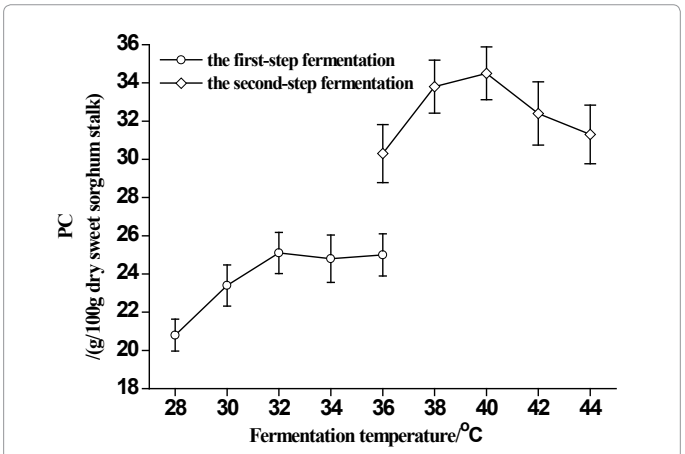

Figure 5: Effect of fermentation temperature on each step fermentation.

The first-step fermentation time/h	PC /(g/100g dry sweet sorghum stalk)	Residual sugar content /(g/100g dry sweet sorghum stalk)
0	2.1±0.1	52.7±1.6
18	18.5±0.7	21.0±0.5
20	23.4±0.9	19.0±0.6
22	26.3±1.1	15.0±0.4
24	27.3±1.2	10.0±0.3
26	28.5±1.2	9.3±0.3

Table 1: Protein and residual sugar content in the product at different fermentation time in the first-step fermentation.

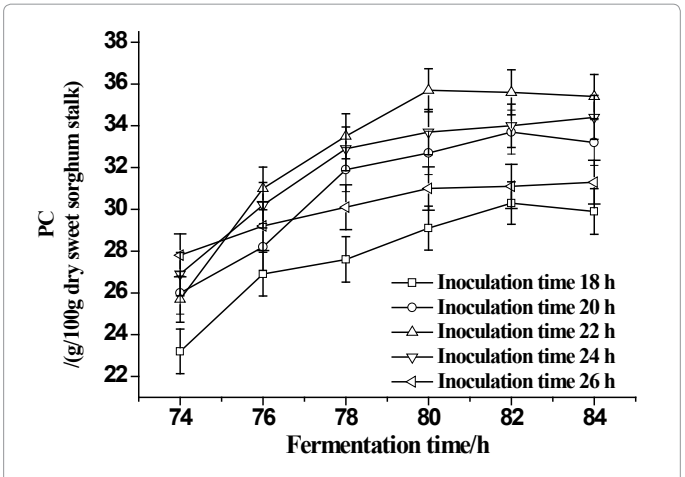

Figure 6: PC at different fermentation time in the second-step fermentation under different inoculation time.

Changes of PC at different fermentation time with initial moisture level of 65±1.31%, natural pH and inoculation amount 6% at 32°C in the first-step fermentation were shown in table 1. It was clear that PC increased and residual sugar decreased quickly at 18h, however, after 22h, PC and residual sugar remained relatively stable.

On the basis of fermentation time in the first-step studied above, *L. rhamnosus* was inoculated after *C. Tropicalis* was cultured for 18h, 20h, 22h, 24h and 26h(inoculation time) at 32°C. *L. rhamnosus* fermentation was carried out with initial moisture level of 70±1.22%, initial pH 6 and inoculation amount 10% at 40°C. Figure 6 showed that when *L. rhamnosus* was inoculated after 22h of the first-step fermentation, the highest PC was eventually obtained after fermentation 80h at 40°C and nearly 100% total sugar was consumed.

Pilot-plant production of protein feed by the two-step solid state fermentation process

According to the fermentation conditions optimized and determined in the above study, the two-step solid state fermentation was applied in pilot scale for protein feed production with sweet sorghum stalks of 3,5000 grams packed in each package of 40 cm×20 cm×80 cm (Figure 7).

The compositions of the fermented product were shown in table 2. The dry matter content in the fermented product was very high, fourfold times as the common silage (5-10%). Crude protein content was not as much as in the flask experiment, but also higher than that in common silage (4%). The high content of lactic acid but low content of butanoic acid ensured good preservation of the product with low toxicity.

Discussion

Effect of addition quantity of $(NH_4)2SO_4$ on *C. tropicalis* fermentation

Sweet sorghum stalk has a high content of sugar (50% or so) which is excellent and low-cost carbon source for yeast. However, it has a low content of nitrogen (2.1%), so $(NH_4)2SO_4$ was added as nitrogen source for *C. Tropicalis* growth. With the increase of added (NH4)2SO4, PC also increased. This is mainly because of the analytical method of N content used in this experiment. But residual sugar content differed, which is a signal of cell growth, as sugar was solely consumed by *C. Tropicalis*. When addition quantity of $(NH_4)2SO_4$ exceeded 1.5%, more residual sugar was detected (Figure 1). This may be because carbon-to-nitrogen ratio in the growth medium was unfitted for *C. Tropicalis* growth. So the addition quantity of $(NH_4)2SO_4$ was chosen to be 1.5% to ensure best growth condition for *C. Tropicalis*.

As *L. rhamnosus* can hardly utilize inorganic nitrogen, it's unnecessary to consider $(NH_4)2SO_4$ in the second fermentation step.

Figure 7: Pilot-plant production of protein feed by the two-step solid state fermentation.

Effect of moisture levels of the substrate on protein content in the product

Solid state fermentation is the enrichment process of strains on the surface of medium in the absence of free water. An optimal moisture level has to be maintained during the fermentation process [21]. Low moisture content reduced the solubility of nutrients of the substrate and limited swelling, and hence inhibited growth of microorganisms; while high moisture level leads to particle agglomeration, gas transfer limitation and competition from bacteria [22,23]. In general, moisture levels in SSF processes vary between 50 and 80%. In addition, aerobic and anaerobic fermentation have different requirements of moisture level. Usually aerobic microorganisms grow better under a lower moisture condition which provides efficient oxygen transfer than anaerobic microorganisms. Therefore, moisture levels of the substrate were investigated respectively in two steps.

The moisture levels of the substrate were tested from 55% to 75%. Within this range, protein content varied a lot in the first step fermentation, but increased with increased moisture levels in the second step. As *C. Tropicalis* is aerobic, moisture level higher than 65% was disadvantageous for oxygen transfer, and thus unfit for *C. Tropicalis* growth. However, *L. rhamnosus* is anaerobic, and oxygen was not preferable for its growth, so higher moisture level was better. But too much water can easily cause agglomeration of the substrate, so the moisture level of 70±1.22% was the best choice.

Effect of pH of the substrate on protein content in the product

The metabolic activities of microorganisms were very sensitive to pH changes, so pH of the substrate should be adjusted beneficial for microorganisms. The natural pH value of sweet sorghum stalk is 4.5-5 which is suitable for growth of yeast, so it is unnecessary to discuss the effect of pH in the first step fermentation. But acidic products produced by *C. Tropicalis* reduced it to 3.5-4 after the first fermentation step. Research showed that suitable pH for *L. rhamnosus* was 6-6.9 [24]. So it was necessary to find the optimal pH value for *L. rhamnosus* before the second fermentation step, and pH 6 was found the best for growth of *L. rhamnosus* (Figure 4). By the way, pH was again reduced to 3.6-4 at the end of the two-step fermentation, due to production of lactic acid and acetic acid.

Effect of incubation temperature on each step fermentation

Incubation temperature is a very important factor for microbial fermentation because of its influence on microbial metabolisms. A slight temperature change may cause big differences. According to the suitable temperatures generally accepted for yeast and lactic acid, fermentation was carried out at 28, 30, 32, 34, 36°C in the first step fermentation and 36, 38, 40, 42, 44°C in the second step. Although the temperature gradient was narrow, the differences were obvious. At 32°C and 40°C respectively, PC was highest.

Dry matter (%)	Crude protein (%)	Acetic acid (mg kg^{-1})	Lactic acid (mg kg^{-1})	Water soluble carbonhydrate (mg kg^{-1})
41.48±1.49	13.77±0.40	8.73±0.10	9.13±0.13	20.60±0.82
Butanoic acid(%)	Neutral detergent fiber(%)	Acid detergent fiber(mg kg^{-1})	pH	Colonies of lactic acid bacteria(×10^5/g)
0.16±0.003	11.48±0.34	7.47±0.28	3.64±0.06	5.5±0.12

Table 2: Compositions of the fermented product by the two-step solid state fermentation.

Determination of fermentation time

C. Tropicalis and *L. rhamnosus* have different demands on culture conditions; therefore, each was single-cultured. Protein content and residual sugar content after the first-step fermentation was very important for the ultimate protein content after the two-step fermentation. If the first step was ended too early, *C. Tropicalis* fails to thrive; but otherwise, sugar in the substrate will be completely consumed, which is insufficient for growth of *L. rhamnosus*. Hence, an optimal fermentation time for a balance of PC and residual sugar at the end of the first step fermentation has to be carefully determined. This couldn't be determined by detecting contents of PC and residual sugar in the product at different ending times, because PC increased and residual sugar content decreased with time (Table 1). Therefore, the interaction between the first-step fermentation time and the second was investigated to get the optimal fermentation time. Figure 6 showed the PC at different combinations of the fermentation time in the first step fermentation and the second step and the highest yield was achieved after 80h with the inoculation time of 22h.

Pilot-plant production of protein feed by the two-step solid state fermentation process

The two-step solid state fermentation process has been successfully applied in Inner Mongolian Autonomous Region, China, by which near 200 tons of the feed was produced from two tons of dry sweet sorghum stalk. Due to the high quality and low price of the feed, it sold very well locally.

Acknowledgements

Financial support to this study was provided by the National Basic Research Program of China (973 Project, No. 2011CB707401), the National High Technology Research and Development Program of China (863 Program, SS2012AA022502), the National Key Project of Scientific and Technical Supporting Program of China (No. 2011BAD22B02).

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