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Production of a water-soluble fertilizer containing amino acids by solid-state fermentation of soybean meal and evaluation of its efficacy on the rapeseed growth

Jianlei Wang, Zhemin Liu, Yue Wang, Wen Cheng, Haijin Mou*

College of Food Science and Engineering, Ocean University of China, 5 Yushan Road, Qingdao 266003, China

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ABSTRACT

Soybean meal is a by-product of soybean oil extraction and contains approximately 44% protein. We performed solid-state fermentation by using *Bacillus subtilis* strain N-2 to produce a water-soluble fertilizer containing amino acids. Strain N-2 produced a high yield of protease, which transformed the proteins in soybean meal into peptide and free amino acids that were dissolved in the fermentation products. Based on the Plackett–Burman design, the initial pH of the fermentation substrate, number of days of fermentation, and the ratio of liquid to soybean meal exhibited significant effects on the recovery of proteins in the resulting water-soluble solution. According to the predicted results of the central composite design, the highest recovery of soluble proteins (99.072%) was achieved at the optimum conditions. Under these conditions, the resulting solution contained 50.42% small peptides and 7.9% poly-γ-glutamic acid (γ-PGA). The water-soluble fertilizer robustly increased the activity of the rapeseed root system, chlorophyll content, leaf area, shoot dry weight, root length, and root weight at a concentration of 0.25% (w/v). This methodology offers a value-added use of soybean meal.

1. Introduction

Soybean (*Glycine max*) is one of the most important crops grown worldwide, and it is cultivated primarily in Argentina, China, America, and Brazil. Global demand for soybean was 255 million tons in 2012, an increase of 1.4% over the previous year (Ministry of Agriculture of the People's Republic of China, 2012. http://pfscnew.agri.gov.cn/fxbg/201207/t20120 711_92917.htm). The main by-products generated during soybean oil production process is soybean meal, which is produced when the oil is extracted from the seed (Rosenthal et al., 2001). Assuming that the efficiency of oil extraction is 19% (w/w), the production of 1 ton of soybean oil results in 4.26 tons of meal. Annual global soybean production in the 2012–2013 season was projected to increase to 188 million tons, and the world's dependence on soybean meal was predicted to increase due to insufficient supply of other meals (United States Department of Agriculture, 2012. http://www.Ers.usda.gov/News/Soybeancoverage.htm). With increased yield, the use of soybean meal has received wide attention. However, the value-added of soybean meal as a by-product has received little attention, leading to a waste of agriculture resources.

Soybean meal contains approximately 44% protein (Bach-Kundsen, 1997), which constitutes a high-quality plant-protein resource. However, traditional thermal processing can destroy the protein quality, making the protein insoluble in water and resistant to disintegration. In order to increase the amount of water-soluble protein or free amino acids, the soybean meal must be hydrolyzed (Bell et al., 1998). Many methods have been used to modify soybean meal protein, such as enzymatic hydrolysis, physical solution, chemical solution and fermentation (Jane et al., 2008). Fermentation has the characteristics of lower consumption and a mild reaction, especially solid-state fermentation (SSF). SSF also requires less equipment and energy, dissolves the structure of soybean meal, and increases the amount of soluble components (Suhartia et al., 2010; Reddy et al., 2008).

It has been reported that the application of rapeseed cake, soybean meal, or other meals as the fertilizer can increase the amount and activity of microbes in soil and improve the quality of plants (Rosiane et al., 2011). Traditional method of composting fermentation meal fertilizer is still used in many parts of China. However, this method has some shortcomings, such as a long fermentation period

Abbreviations: SSF, solid-state fermentation; WSF, water-soluble fertilizer; TN, total nitrogen; TC, total carbohydrate; RWP, recovery of water-soluble protein.

* Corresponding author. Tel.: +86 532 82032290.
E-mail address: mousun@ouc.edu.cn (H. Mou).

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(one to six months) and a high loss of nitrogen (nearly 30–50% during the process of composting) (Xu et al., 2000). Thus, nitrogen cannot be used effectively and sufficiently for plant growth, making the use of meal for this purpose wasteful. The use of a strain that produces a high yield of proteases in SSF can make up for these shortcomings (Pandey, 2003). SSF by microorganisms has been widely used to hydrolyze soybean meal protein (Kuo et al., 2006; Terlabie et al., 2006). Conditions during the SSF process are difficult to control. This has resulted in the restriction of large-scale industrialization of SSF (Holker et al., 2004; Mitchell et al., 2003; Xiao et al., 2007). It is critical to optimize the parameters of the SSF process for the production of a high-quality fertilizer.

Fertilizer, especially water-soluble fertilizer (WSF) containing free amino acids, has been widely used for promoting the growth of plants and improving their quality. It also reduces the required dose of pesticides and protects the ecological environment.

The strain *Bacillus subtilis* N-2, which produces a high yield of protease, was newly isolated and used to produce water-soluble fertilizer containing amino acids through solid-state fermentation of soybean meal. This comprehensive study was conducted to optimize the fermentation process for the production of WSF. The effects of the resulting fertilizer were tested in a rapeseed pot experiment.

2. Materials and methods

2.1. Materials

Soybean meal was purchased from Shandong Bohi Co., Ltd (Qingdao, China). The properties of the soybean meal are shown in Table 1. Rapeseed was purchased from Shandong Research Institute of Vegetables (Jinan, China).

2.2. Microorganism and sequencing of 16s rDNA

A strain with high protease activity was isolated from soil and named as N-2, and it is preserved by the laboratory of Applied Microbiology, Ocean University of China. The strain was cultured on nutrient agar medium (NA: 3 g/L beef extract, 10 g/L peptone, 5 g/L sodium chloride, 18 g/L agar and pH 7.0) and kept at 4 °C.

N-2 was inoculated into nutrient broth and aerobically grown on a shaker (32 °C, 170 rpm) for approximately 36 h. Genomic DNA was extracted and purified from the culture using a genome DNA extraction kit and used as the template for polymerase chain reaction (PCR) (Sangon, Shanghai, China).16S rDNA was amplified with the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1,492R (GGCTACCTTG TTACGACTT) in a DNA thermal cycler (Applied Biosystems 2720) and then sequenced (Sangon, Shanghai, China). A phylogenetic tree was constructed using MEGA 5.0 based on the DNA alignment produced by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. SSF

The N-2 inoculum was prepared by incubating N-2 into nutrient broth and cultivating the broth at 32 °C for 36 h. Unless otherwise stated, solid-state fermentation (SSF) was carried out as follows. Soybean meal was thoroughly mixed with the liquid (liquid: 10 g/L glucose, 3 g/L beef extract, 2 g/L sodium chloride, 1 g/L K_2HPO_4, 1 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $FeSO_4{\cdot}7H_2O$, pH 7.0) by mixer until no significant amount of water was visible. One hundred grams of each mixture was put into a 500 mL flask for SSF and sealed with Parafilm (Penchiney Plastic Packaging, Chicago, USA) to prevent the evaporation of the water while permitting the exchange of air. Ten percent inoculum was added to each flask. Each flask was mixed carefully under strictly aseptic conditions with sterile glass rods. The flasks were incubated in a chamber with a relative humidity above 50% at 32 °C for 12 days. Cultures were kept static when not stirring.

2.4. Assay for proteolytic activity

After solid-state fermentation, 5 g of freeze-dried sample was extracted by 100 mL of water for the detection of proteolytic enzyme. The sample was treated in a shaking bath at 40 °C, 150 rpm for 1.0 h, then centrifuged at 4000 rpm for 20 min. The supernatant was tested for the enzyme activity.

Protease activity was tested according to the reported method (Takami et al., 1989; Tari et al., 2006). One protease unit was defined as the enzyme amount that could produce one milligram of tyrosine in one minute under the defined assay conditions.

2.5. Extraction and chemical analysis of water-soluble components

Fermentation samples were extracted by adding 30 mL of water to the 10 g of solid substrate material. The samples were blended with a rotator at 150 rpm for 0.5 h so that no lumps of mycelium or substrate remained. Each sample was extracted three times and filtered through 200 mesh bolting cloth (Hengfa Textile Co., Ltd., Anhui, China). The solution was dried using a rotary vacuum evaporator and then used as fertilizer.

The total nitrogen (TN) in the water-soluble solution was determined by Kjeldahl^{TM} 8400 (FOSS Co. Ltd., Sweden) (Miler and Houghton, 1945). The small peptide content was expressed as a trichloroacetic acid-nitrogen solution index (TCA-NSI) (Yoshitsugu et al., 1999). The total carbohydrate (TC) was determined by the phenol-sulfuric acid method (Dubois et al., 1956). The macronutrients potassium and phosphorus were determined using an inductively coupled plasma optical emission spectrometer (710 ICP-OES, Varian Technologies, USA). An amino acid analyzer (L-8900 System: Hitachi Inc.) equipped with a visible detector was used to analyze individual amino acid content (Zhang et al., 2014). The amino acid content was expressed as g/100 g of the water-soluble fertilizer weight. The amount of γ-PGA was determined using SBA-60 biosensor analysis (Goto and Kunioka, 1992).

The recovery of water-soluble protein (RWP) was defined using the following equation:

$$\mathrm{RWP} = \left(\frac{P_{ws}}{P_{tot}}\right) \times 100\% \quad (1)$$

where P_{ws} is the amount of protein in the solution and P_{tot} is the total amount of protein as determined by Kjeldahl method.

2.6. Plackett–Burman design

The initial pH of the fermentation substrate, number of days of fermentation, ratio of soybean meal to liquid, frequency of agitation, and inoculum content were selected as the five main variables for the Plackett–Burman design. According to the characteristics of SSF and the results of single factor experiments, a high level and low level for each factor were determined. The experimental design is shown in Table 2. According to the 2-level, 5-variable approach, a 2^{5-2} design consisting of 20 factorial runs was carried out. Statistical significance was determined at the 95% confidence level ($p < 0.05$) using Minitab 15.0 software (State College, PA, USA).

2.7. Central composite design

Three significant factors (fermentation days, initial pH of the fermentation substrate, and ratio of soybean meal to liquid) were selected as main variables as identified by the Plackett–Burman