

# Protein Enrichment of Sweet Potato Beverage Residues Mixed with Peanut Shells by *Aspergillus oryzae* and *Bacillus subtilis* Using Central Composite Design

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Received: 14 April 2016 / Accepted: 28 January 2017 / Published online: 8 February 2017  
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## Abstract

**Purpose** The present study aimed at reducing the pollution of the waste generated by the sweet potato beverage industry to the environment and transforming the residues mixed with peanut shells into biomass protein to be used as animal feed.

**Method** Six different microbial strains were evaluated for their ability to produce true protein using the mixed substrates as a nature medium under solid-state fermentation.

**Results** The experimental results revealed that the highest true protein content was obtained when the substrates were fermented with a combination of *Aspergillus oryzae* and *Bacillus subtilis*. The optimal process parameters for protein enrichment by solid-state fermentation using *A. oryzae* and *B. subtilis* through Central Composition Design (CCD) included initial moisture content of 63.7%, incubation of temperature 26.9 °C and fermentation time of 67.5 h. Expression profile of protein system was characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fermentation products appeared as five major protein bands, indicating that the protein components had increased after solid-state fermentation by *A. oryzae* and *B. subtilis*.

**Conclusion** This study developed an efficient and reliable fermentation method to utilize the industrial wastes of sweet potato beverage residues and peanut shells for the animal protein feed.

**Keywords** Sweet potato beverage residues · Peanut shells · Central composite design · *Aspergillus oryzae* and *Bacillus subtilis* · SDS-PAGE · Protein enrichment · In vitro digestibility

## Introduction

The increasing global demand for food and animal feed protein has stimulated the search for non-conventional sources of protein to supplement existing resources [1]. Recently, there has been considerable interest regarding the biotechnological potential of converting agro-industrial wastes into protein feed. Solid-State fermentation (SSF), requires less energy and does not generate as much effluent as other related technologies. It ultimately represents a more environmentally friendly process to generate single-cell proteins [2], detoxify cottonseed meal [3] and enrich proteins from apple pomace [4], discarded oranges [5] and potato starch residues [6].

Microorganisms used in SSF include fungi, yeasts, bacteria, and algae. Fungi, especially filamentous fungi such as *Aspergillus niger* and *Aspergillus oryzae*, are widely used in fermented foods. *Trichoderma reesei* has been generally used to treat lignocellulosic plant biomass because of its ability to secrete cellulases. Yeasts have been used to produce single-cell proteins with their abundant mycoprotein content. Some beneficial *Bacillus* species are able to secrete cellulases and amylases, and can also produce broad spectrum antibiotics. Currently, co-culture SSF (mostly positive interactions) is widely used because the colonization, penetration, and degradation of the solid substrate depend on a symbiotic association. This occurs where each species can function synergistically with the other strains in a substrate niche through a specific set of enzymes [7]. There have

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been several studies about SSF involving fungal co-cultures or yeast co-cultures with bacteria for enzyme and protein [8–12]. However, it was a pity that rarely research on SSF with filamentous fungi co-cultured with beneficial bacteria for protein enrichment.

According to the Food and Agriculture Organization of the United Nations, the annual production of sweet potato in China is about  $7.56 \times 10^7$  tonnes [13]. Recently, a healthy sweet potato beverage has been developed as a new type of drink that is popular among consumers of all ages. However, the production of this drink has generated a sea of residues rich in water, starch, cellulose and a small amount of protein. The residues had a low effective of feed conversion when feeding the animals directly due to the unbalance of nutrition content. In addition, varies of bacteria, fungi, love to land it and reproduction because of the high moisture content, some of them even secrete toxins that unsafe for the animals, so numerous sweet potato beverage residues (SPBR) were open dumped, the disgusting smell and effluent waste water had a tremendous negative effect on the environment.

Additionally, the increasing expansion of peanut production has led to accumulation of large quantities of peanut shells (PS) all over the world. Globally, 45.6 million metric tons of peanuts are produced annually. It has been estimated that for every kg of peanuts produced, 230–300 g of PS are generated. Therefore, as much as 13.7 million metric tons of PS are churned out every year, most of which are discarded or burned as garbage [14]. This also leads to wasted resources and contributes to environmental pollution. Alternatively, pulverized PS can be used to adjust the water content of SPBR, which not only loosens the substrate, making it better for SSF but also takes full advantage of PS as a natural resource.

Small fractions of PS were incorporated into animal feed, especially for the ruminants [14]. Sweet potato wastes also have been fermented into feed for poultry and ruminants in many Asian countries [15], so the mixture of SPBR and PS has certain feasibility as the animal feed. However, the high content of lignocellulose of the PS as well as urea and  $(\text{NH}_4)_2\text{SO}_4$  will be added in the substrates made the production better applied for the ruminants. The nutrition value is achieved mainly by increasing its protein and lowering its carbohydrate content. Nutritional evaluation of the final fermented products (FFP) was achieved mainly by estimation of its protein degradability in the rumen of cattle. The degradable fraction of protein in the rumen is converted mainly to ammonia, which is being used for microbial protein synthesis, while the undergradable fraction becomes available for digestion and absorption in the intestine [16].

An effective alternative to factorial design is Central Composite Design (CCD), originally developed by Box

and Wilson and improved upon by Box and Hunter [17]. Though it was said CCD needed higher experimental run and complex calculation than the BBD (Box-Behnken Design) [18], CCD is better than BBD experiment on response surface fitting and has been shown to be sufficient to describe the majority of steady-state process responses [19–21]. Hence in this study, it was decided to use CCD to design the effects of initial moisture content, temperature and fermentation time on the true protein (TP) content during SSF.

## Materials and Methods

### Substrates

Sweet potato beverage residues were obtained from a local juice factory in Shijiazhuang, China, and used within 12 h of production. Peanut shells were collected from a local peanut husking factory in Shandong, China, and pulverized to an average particle size of 1 mm.

### Microorganisms and Their Propagation

*Aspergillus niger* CGMCC-3.01858, *T. reesei* CGMCC-3.3711 and *Candida utilis* CGMCC-2.1012 used in this study were obtained from the China General Microbiological Culture Collection Center. *A. oryzae* (accession number: KU320673), *Saccharomyces cerevisiae* (accession number: KM005256), and *B. subtilis* (accession number: KU239979) were isolated from total mixed rations silage of our laboratory. Moreover, it was testified that *B. subtilis* can secrete amylase using the method of Lugol's iodine staining. The fungi and yeasts were maintained on Potato Dextrose Agar (PDA) slants, while *B. subtilis* was grown on nutrient broth (NB) slants. All microbes were stored at 4 °C. The fungi and yeasts were activated on PDA slants incubated at 28 °C for 2–5 days, and fungal spore suspensions were prepared from PDA plates. *B. subtilis* was activated on NB slants incubated at 37 °C for 2 days.

To prepare organism suspensions, Potato dextrose broth (PDB) and NB (without agar) were dissolved in distilled water, blended well, divided into test tubes, autoclaved at 121 °C for 15 min at 15 psi and then cooled to room temperature. Separate test tubes containing PDB were inoculated with pure yeast strains, and incubated at 28 °C for 24 h. Similarly, individual test tubes with NB were inoculated with *B. subtilis*, and incubated at 37 °C for 24 h. To prepare spore suspensions, fungal cells were cultured on PDA plates at 28 °C for 5 days to attain a high-density of conidia. Spores were collected from plates by gentle washing with Mandel's medium [22] to obtain spore suspensions

consisting of  $10^7$  spores/mL. Spore suspensions were stored at 4 °C until used.

To test the compatibility of fungal and bacterial strains, agar plates containing SPBR and PS were prepared. A 100-g sample of SPBR was dried in a forced-air oven at 65 °C for 48 h and then milled to a 20–40 mesh size. Culture medium was prepared by dissolving 1.5% SPBR powder, 1.5% PS and 2% agar in water, autoclaving at 121 °C for 15 min at 15 psi and then pouring into individual plates.

### SSF of the Substrates Using a Single Strain

The SSF medium for microbial protein production was carried out in 500-mL Erlenmeyer flasks consisted of 38.5 g fresh SPBR, 11.5 g PS, 0.5 g urea, 0.5 g  $(\text{NH}_4)_2\text{SO}_4$ , and 0.5 mL of Mandel's medium with slight modification which contained the following chemicals (%):  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{CaCl}_2$  4.5,  $\text{KH}_2\text{PO}_4$  20,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.05,  $\text{CoCl}$  0.02,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  0.016 and  $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$  0.014. The medium was sterilized at 121 °C for 20 min at 15 psi before being inoculated. All flasks for experiments with single or mixed strains were inoculated with a 10% spore and/or cells suspension ( $10^7$ – $10^8$  spores or cells). The cultures were thoroughly mixed with a sterile glass rod and the flasks were sealed with sealing membrane. The flasks were then incubated without agitation at 30 °C and 65% relative humidity for 72 h. At the end of the fermentation period, the products were dried at 65 °C for 48 h, and then pulverized for compositional analyses. Experiments were completed with three replicates.

### SSF of the Substrates with Different Microbial Combinations

*Aspergillus oryzae*, *T. reesei*, *S. cerevisiae* and *B. subtilis* were used in the following combinations for SSF to investigate their effects on TP content: (1) *A. oryzae* + *S. cerevisiae*, (2) *A. oryzae* + *B. subtilis*, (3) *T. reesei* + *S. cerevisiae*, (4) *A. oryzae* + *T. reesei* + *S. cerevisiae*, (5) *A. oryzae* + *T. reesei* + *B. subtilis*, (6) *A. oryzae* + *S. cerevisiae* + *B. subtilis* and (7) *A. oryzae* + *T. reesei* + *S. cerevisiae* + *B. subtilis*. The protein contents of the substrates with an initial moisture content of 60% were analyzed after SSF at 30 °C for 72 h. Each combination was performed with three replicates.

### Compatibility Tests

After SSF using mixed strains, we compared the compatibilities of *A. oryzae* with *S. cerevisiae* and *A. oryzae* with *B. subtilis*. Single and pairwise combinations of strains were grown on PDA and SPBR/PS agar plates. Spore suspensions of *A. oryzae*, organism suspensions of

*S. cerevisiae*, and *B. subtilis* cells were separately diluted to  $10^{-5}$ . Three PDA plates and three SPBR/PS agar plates were individually inoculated with 0.1 mL diluent, which was spread over the entire plate surface. An additional two PDA plates and two SPBR/PS plates were inoculated with a mixture of 50  $\mu\text{L}$  diluted *A. oryzae* spore suspension and 50  $\mu\text{L}$  diluted *S. cerevisiae* culture fluid or a mixture of 50  $\mu\text{L}$  diluted *A. oryzae* spore suspension and 50  $\mu\text{L}$  diluted *B. subtilis* NB culture. Plates were incubated at 30 °C for 7 days. Fungal compatibility was based on colony shapes, radial expansion and growth pattern as described by Hu et al. [23] and Masoud et al. [24]. The plates were observed on each day of incubation.

### Experimental Design for Optimization of SSF Process Parameters

The technical parameters of inoculation ratio, inoculation method, and total inoculum size of the substrates on the TP content were based on a published study [6]. The RSM was employed to optimize the conditions for TP production using Design-Expert, version 8.0.5 b (State-Ease, Inc., Minneapolis, MN, USA). A three-level-three-factor central composite design (CCD) was performed to determine the best combination of fermentation variables for optimizing the yields of TP. Six replicates at the center of the design were used to estimate the pure error sum of squares. The variables were defined as initial moisture content ( $X_1$ ), temperature ( $X_2$ ) and fermentation time ( $X_3$ ). The response variable was TP content ( $Y$ , %). The uncoded and coded levels of the independent variables used in the CCD are listed in Table 1. The experimental data were regressed according to the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j \quad (1)$$

where  $\beta_0$  is the value of the fitted response at the center point of the design (0, 0, 0), and  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$ , and  $\beta_{ij}$  are the constant, linear, quadratic, and cross-product regression terms, respectively.

### Protein Profile According to SDS-PAGE Analysis

Crude extracts from the unfermented and fermented substrates were prepared by adding 150 mL 50 mM citrate buffer (pH 4.8) to the contents of each flask and shaking the contents at 150 rpm for 30 min. The protein broth was filtered using four layers of gauze, and the resulting filtrate was centrifuged at  $10,000 \times g$  for 15 min at 4 °C. Crude extracts were immediately used for SDS-PAGE carried out as described by Laemmli [25], using 12.5% (w/v) separation gel. Protein bands were visualized by staining with

**Table 1** Central composite design and the response for the TP content of the fermented products

Run	SSF parameters <sup>a</sup>			Protein contents (%, w/w)	Increase in protein contents (%) after SSF <sup>b</sup>
	$x_1$ ( $X_1$ )	$x_2$ ( $X_2$ )	$x_3$ ( $X_3$ )	$Y$	$\Delta Y$ (%, w/w)
1	-1 (50)	-1 (20)	-1 (48)	9.92	52.85
2	1 (70)	-1 (20)	-1 (48)	8.81	35.75
3	-1 (50)	1 (30)	-1 (48)	10.30	58.71
4	1 (70)	1 (30)	-1 (48)	14.69	126.35
5	-1 (50)	-1 (20)	1 (96)	10.8	66.41
6	1 (70)	-1 (20)	1 (96)	9.38	44.53
7	-1 (50)	1 (30)	1 (96)	7.96	22.65
8	1 (70)	1 (30)	1 (96)	13.8	112.63
9	-1.68 (43.2)	0 (25)	0 (72)	7.01	8.01
10	1.68 (76.8)	0 (25)	0 (72)	10.15	56.39
11	0 (60)	-1.68 (16.6)	0 (72)	7.87	21.26
12	0 (60)	1.68 (33.4)	0 (72)	10.01	54.24
13	0 (60)	0 (25)	-1.68 (36)	11.93	83.82
14	0 (60)	0 (25)	1.68 (112.4)	11.12	71.34
15	0 (60)	0 (25)	0 (72)	14.88	129.28
16	0 (60)	0 (25)	0 (72)	15.96	145.92
17	0 (60)	0 (25)	0 (72)	14.95	130.35
18	0 (60)	0 (25)	0 (72)	16.41	152.85
19	0 (60)	0 (25)	0 (72)	15.03	131.59
20	0 (60)	0 (25)	0 (72)	14.88	129.28

<sup>a</sup> $X_1$ =initial moisture content (in percent, w/w),  $X_2$ =temperature (°C), and  $X_3$ =fermentation time (in hour)

<sup>b</sup> $\Delta Y = (Y - 6.49) \times 100 / 6.49$ . The value 6.49% (w/w) was the protein content of the substrates without fermentation

Coomassie brilliant blue R-250. The protein standards (TaKaRa, Dalian, China) used for molecular mass calibration were phosphorylase b (97.2 kDa), albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), and  $\alpha$ -lactalbumin (14.4 kDa).

### In Vitro Digestibility Determination

In vitro digestibility of DM (DMD), OM (OMD), CP (CPD), NDF (NDFD) and ADF (ADFD) were determined using the method of Tilley and Terry [26]. In brief, 0.5 g air-dried untreated and fermented samples were weighed into Nylon filter bags (Ankom F57, ANKOM Technology, Fairport, New York) and sealed. Rumen cannulated steers fed a basal diet was used in the in situ study to supply inoculum for the in vitro digestibility study [27]. Rumen fluid of steers fed a corn silage based diet was mixed with buffer solution under anaerobic conditions. The samples with filter bags were incubated in 40 ml buffered rumen fluid for 48 h at 39 °C. After 48 h of incubation, filter bags were removed from the tubes and washed with distilled water. Standard procedures were followed in the calculation of DM, OM, CP, NDF and ADF digestibility.

### Analytical Method

Moisture content (% w/w) was determined by weighing samples before and after drying at 65 °C in a hot air oven for 48 h. The TP was precipitated with trichloroacetic acid and then measured according to the Kjeldahl method (% DM) [28]. Total soluble carbohydrates (TSC) was analysed using the automated procedure method [29]. Crude fat or ether extract (EE) was determined by the Soxhlet extraction method according to AOAC [30]. Ash-free neutral detergent fiber (NDF) was analyzed by a modified method of Van Soest et al. [31] with addition of a heat stable amylase, and ash-free acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed by the method of Van Soest [32]. The content of hemicellulose was calculated as the difference between NDF and ADF and cellulose as the difference between ADF and ADL. The FFP was tested for aflatoxins  $B_1$ ,  $B_2$ ,  $G_1$ ,  $G_2$  and the other mycotoxines according to Almeida [33]. Nitrogen-free extract (NFE) content was calculated according to the following model:

$$NFE = 100 - (CP + EE + CF + CA) \quad (2)$$

In which, All terms are expressed as % of dry matter (DM).

### Statistical Analysis

All data were presented as the mean  $\pm$  standard deviation from triplicate measurements. Data were analyzed using SPSS, version 21.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was performed. The significance of differences between mean values was assessed using the LSD test and Duncan's Multiple Range ( $p < 0.05$ ). The results of optimization by RSM were analyzed using Design-Expert, version 8.0.5 b.

## Results and Discussion

### Composition of the Substrates

No aflatoxins or other mycotoxins were detected in all samples tested (unfermented substrates and FFP). Table 2 presents the proximate composition of the substrates used in the protein enrichment by SSF. It can be seen that the substrates had a moisture content of approximately 60%, were rich in water-soluble carbohydrates (25.99% DM), but had low protein contents (6.49% DM). This natural medium is abundant in sugar and appears to be an excellent substrate for bioprocesses. Additionally, SPBR and PS are inexpensive and available in large amounts during the harvesting season. Therefore, several microorganisms can use these residues as natural substrates.

What's more, it can be seen that the ash and fat content did not change a lot before and after fermentation. However, TSC content has a significant decrease after fermentation, which is may be due to the utilization of the TSC for *A. oryzae* and *B. subtilis* grow on the substrate and synthesis of protein. The crude fiber content is 51.12% after fermented, which has a slightly decreased due to lignin content decreased (from 19.92% DM to 16.89% DM), cellulose and hemicelluloses content of the substrate was almost unchanged, this is different with the result of Iconomou

et al [34], in which research, the fermentation by *Trichoderma viride*, *Phanerochaete chrysosporium*, *Dichomitus squalens* and *Phlebia radiata* increased the protein content of sugar beet pulp by 70.0%, mainly at the expense of hemicellulose, which decreased by 54.0% (from 36.7 to 16.7% DM), while cellulose and lignin was slightly reduced, which indicated that different strains have different capacity as well as the substrates also make a difference.

### Effect of SSF on TP Content of the Substrates

The TP contents of the substrates following single-strain fermentation with various microbes are presented in Fig. 1a. The substrates inoculated with *A. oryzae* had the highest TP content (13.78%), suggesting this fungus may be useful for increasing the content of TP in substrates. This result was consistent with the findings of a previous study in which protein content increased from 50.47 to 58.93% (w/w) after 36 h of fermenting soy meal, significantly enhancing the content of amino acids [35].

The effects of different strain combinations on TP content are shown in Fig. 1b. The substrates fermented with *A. oryzae* and *B. subtilis* had a significantly higher TP content than the substrates fermented with other microbial combinations. These results differed from those of previous studies [36–38], which reported higher protein contents could be achieved using a combination of fungus and yeast. Only one study has evaluated the effects of the combination of *Geotrichum candidum* and *Bacillus subtilis* on fermenting rapeseed meal, wheat bran, corn flour, and rice bran. The reported protein yield was only 12.8%. Additionally, these raw materials are relatively expensive [39].

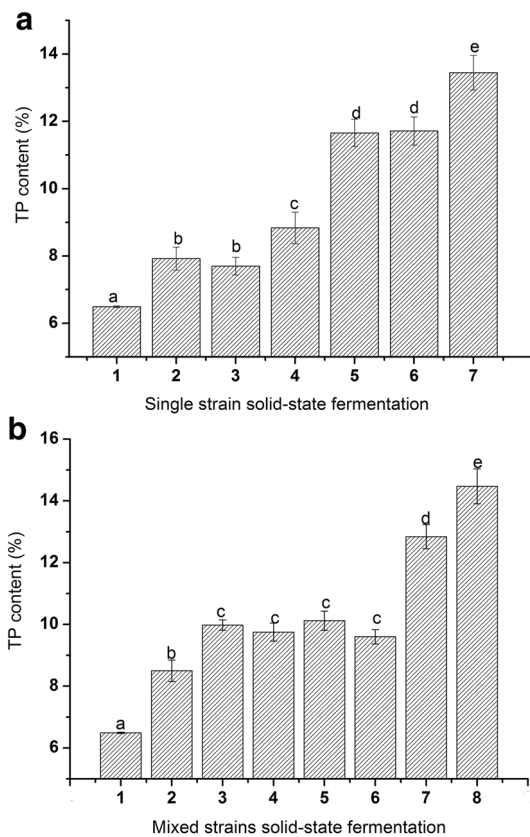
More importantly, *A. oryzae* and *B. subtilis* are not only micro-organisms that are permitted as feed additives, they are also beneficial to humans and animals. Although fermenting with *B. subtilis* alone did not increase protein content, it likely has a considerable synergistic effect with *A. oryzae*. During fermentation, white mycelium was observed covering the substrates after 48 h fermentation with the combination of *A. oryzae* and *B. subtilis*, indicating these microbes grow rapidly. This observation may be

**Table 2** Proximate composition of SPBR, PS, the mixed substrates unfermented and fermented

Com-position (%w/w)	DM (%)	aNDF	ADF	Cellulose	Hemi-cellulose	Lignin	CP	TP	TSC	Ash	Fat	Crude fiber	NFE
SPBR	26.73	14.41	10.48	5.92	3.93	4.56	8.60	7.14	41.38	2.51	2.71	24.51	61.67
PS	92.83	76.00	66.90	38.75	9.10	28.15	4.47	4.36	10.60	3.25	1.20	76.59	14.49
US	41.93	52.83	44.57	24.65	8.26	19.92	14.76	6.49	25.99	2.88	2.00	53.47	26.89
FFP	35.64	50.71	43.13	26.24	7.58	16.89	21.91	15.58	5.12	3.43	2.06	51.12	21.48

DM dry matter, aNDF neutral detergent fiber, ADF acid detergent fiber, CP crude protein, TSC total soluble carbohydrates, NFE nitrogen free extracts, US unfermented substrates same as the SSF medium without inoculation, FFP final fermented products





**Fig. 1** Effect of SSF on TP content of the fermented substrates. **a** Single-strain SSF (1, control; 2, *C. utilis*; 3, *B. subtilis*; 4, *S. cerevisiae*; 5, *T. reesei*; 6, *A. niger*; 7, *A. oryzae*). **b** Mixed-strains SSF (1, control; 2, *A. oryzae* + *S. cerevisiae*; 3, *A. oryzae* + *T. reesei* + *S. cerevisiae* + *B. subtilis*; 4, *T. reesei* + *S. cerevisiae*; 5, *A. oryzae* + *T. reesei* + *S. cerevisiae*; 6, *A. oryzae* + *S. cerevisiae* + *B. subtilis*; 7, *A. oryzae* + *T. reesei* + *B. subtilis*; 8, *A. oryzae* + *B. subtilis*). Values on the columns with different letters (a, b, c, d, e) are significantly different ( $p < 0.05$ ) according to LSD test and Duncan's Multiple Range Test

due to two main factors. First, *A. oryzae* has greater glucoamylase and cellulase activities, which enable the degradation of cellulose during substrate fermentation to produce available carbon source. Second, the *B. subtilis* strain used in this article can secrete amylase, which accelerates starch degradation and promotes the mycelial growth of *A. oryzae*. Further research is required to confirm these two factors are responsible for the observed results.

### Compatibility Analysis

In this study, the compatibilities of the *A. oryzae* and *S. cerevisiae* co-culture and the *A. oryzae* and *B. subtilis* co-culture were tested by co-cultivating them pairwise on PDA and SPBR/PS agar plates. In general, the growth of each strain was affected by the presence of the other strain on the same plate. The *S. cerevisiae* colonies were much smaller in the *A. oryzae* zone of growth than on plates inoculated with *S. cerevisiae* alone. However, *B. subtilis* colonies were unaffected by the presence of *A. oryzae*, and grew better on SPBR/PS agar plates than on PDA. Additionally, *A. oryzae* produced spores more slowly in the presence of *B. subtilis* than when *A. oryzae* was cultured alone on both plates. These results confirmed that *B. subtilis* has a synergistic effect with *A. oryzae* co-culture on the SPBR/PS substrate.

### Effects of Optimization of Technical Parameters on TP Content

The effects of inoculation ratio, inoculation method, and total inoculum size of the substrates on the TP content are described in Tables 3 and 4. The results indicated that a 72-h fermentation using 5-mL *A. oryzae* and *B. subtilis* co-culture at 1:1 inoculation proportion can increase TP content by up to 14.47%.

**Table 3** The effect of inoculation ratio and method on TP content of the substrates fermented by mixed strains

Ratio and method	Control	1:1	1:2	2:1	A	B	C
TP (%)	6.49 <sup>a</sup>	14.47 <sup>b</sup>	11.76 <sup>c</sup>	12.07 <sup>c</sup>	14.47 <sup>b</sup>	13.1 <sup>c</sup>	13.4 <sup>c</sup>

The order of the ratio of both strains was *A. oryzae*: *B. subtilis*. A: Inoculation the two strains at the same time; B: *A. oryzae* fermentation 36 h then inoculation *B. subtilis*; C: *B. subtilis* fermentation 36 h then inoculation *A. oryzae*; Total fermentation time is 72 h

<sup>a-c</sup>Means in the same row with different superscripts differ ( $p < 0.05$ )

**Table 4** The effect of inoculation size on TP content of the substrates fermented by mixed strains

Inoculation size (mL)	0	3	5	8	10
TP (%)	6.49 <sup>a</sup>	13.77 <sup>b</sup>	14.47 <sup>b</sup>	12.22 <sup>c</sup>	11.30 <sup>d</sup>

<sup>a-d</sup>Means in the same row with different superscripts differ ( $p < 0.05$ )

### Effects of Optimization of SSF Parameters by RSM on TP Content

When many factors and interactions affect the desired responses, RSM is an effective tool for optimizing the process. RSM uses an experimental design such as the CCD to fit a model using the least-squares technique. If the proposed model is adequate, as revealed by the diagnostic tests provided by an analysis of variance (ANOVA) and residual plots, contour plots can be usefully employed to study the response surface and locate the optimum [40]. A multiple regression analysis of the experimental data yielded the following equation:

$$Y = 15.31 + 0.95 \times X_1 + 0.84 \times X_2 - 0.23 \times X_3 + 1.6X_1X_2 + 0.14X_1X_3 - 0.58X_2X_3 - 2.11X_1^2 - 1.98X_2^2 - 1.06X_3^2 \quad (3)$$

where *Y* is the yield of TP, *X*<sub>1</sub>, *X*<sub>2</sub>, and *X*<sub>3</sub> are the coded variables for initial moisture content, temperature and fermentation time, respectively.

The ANOVA results are provided in Table 5. The model’s *F*-value of 20.10 indicated that the model was significant (*p*<0.01). The lack-of-fit was not significant (*p*>0.05). The *R*<sup>2</sup> value (multiple correlation coefficient) close to 1 denotes a good correlation between the experimental and predicted values. In this case, the value of *R*<sup>2</sup> was 0.9476, indicating a good correlation between the experimental and predicted values.

The linear terms of initial moisture content, temperature, and the quadratic terms of all the independent variables were significant according to the *F* test (*p*<0.05).

**Table 5** Analysis of variance for the response surface quadratic model of the protein content

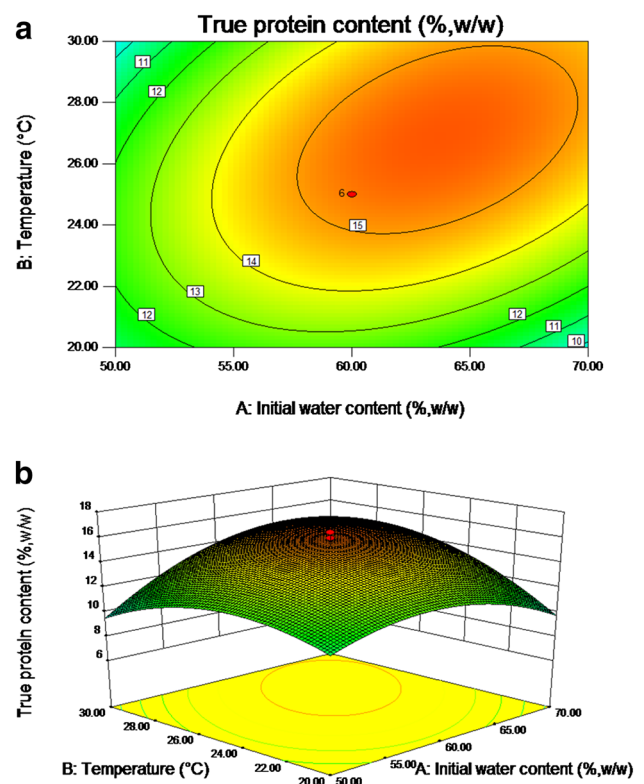
Source	Sum of squares	DF	Mean square	F value	<i>P</i> values
Model	162.38	9	18.04	20.10	<0.0001
<i>X</i> <sub>1</sub>	12.34	1	12.34	13.75	0.0041
<i>X</i> <sub>2</sub>	9.58	1	9.58	10.68	0.0085
<i>X</i> <sub>3</sub>	0.72	1	0.72	0.81	0.3905
<i>X</i> <sub>1</sub> <i>X</i> <sub>2</sub>	20.35	1	20.35	22.68	0.0008
<i>X</i> <sub>1</sub> <i>X</i> <sub>3</sub>	0.16	1	0.16	0.18	0.6795
<i>X</i> <sub>2</sub> <i>X</i> <sub>3</sub>	2.74	1	2.74	3.05	0.1113
<i>X</i> <sub>1</sub> <sup>2</sup>	63.86	1	63.86	71.16	<0.0001
<i>X</i> <sub>2</sub> <sup>2</sup>	56.37	1	56.37	62.81	<0.0001
<i>X</i> <sub>3</sub> <sup>2</sup>	16.31	1	16.31	18.17	0.0017
Residual	8.97	10	0.90		
Lack of fit	6.78	5	1.36	3.08	0.1212
Pure error	2.20	5	0.44		
Cor total	171.36	19			

*DF* degree of freedom; *R*<sup>2</sup>=0.9476, *R*<sup>2</sup>(adj)=0.9005. Cor total: totals of all information corrected for the mean

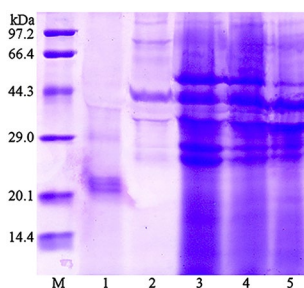
There was a significant interaction between initial moisture content and temperature that affected protein content. Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models were chosen for the axes of contour plots to account for curvature of the surfaces. In Fig. 2, the temperature and initial moisture content were selected as the vertical and horizontal axes respectively for the contour plot and 3 D-surface of TP yield employed by Design expert 8.0.6. These results revealed that initial moisture content and temperature exhibited a profound effect on protein production.

### Optimization

The model is useful in indicating the direction in which to change variables in order to maximize the TP yield (*Y*). According to the analysis using Design Expert 8.0.6 software, the following conditions can be recommended as a practical optimum: initial moisture content 63.7%, temperature 26.9 °C, and fermentation time 67.5 h. The optimal fermentation temperature (26.9 °C) is ideal because it coincides with the ambient temperature during the season of SPBR effluent. The estimated TP yield was 15.67%. A verification experiment at the optimum conditions, consisting



**Fig. 2** Contour plots (a) and response surface (b) for the effect of Initial moisture content and temperature on TP yield



**Fig. 3** SDS-PAGE pattern of protein profiles of unfermented substrate and fermented products under 72-h fermentation in static flask bioreactor. M Molecular mass markers, lane 1, protein band patterns of the unfermented substrate, 2–5 protein band patterns of the different microbial combination fermented products. Lane 2, *A. oryzae* + *S. cerevisiae*, lane 3, *A. oryzae* + *B. subtilis*, lane 4, *A. oryzae*, lane 5 *A. oryzae* + *T. reesei* + *B. subtilis*, sample load volume, 10  $\mu$ L

of three runs, was performed and the practical TP yield, Y, was  $15.58 \pm 0.99\%$ .

### SDS-PAGE Analysis

The SDS-PAGE protein profiles of the unfermented and fermented substrates are presented in Fig. 3. The results showed that the patterns of protein bands from the unfermented substrates were concentrated in the low and moderate molecular weight regions from 20.1 to 25 kDa (lane 1). The protein bands for the fermented substrates were mainly observed in higher molecular weight regions (i.e., 27–55 kDa). Protein bands around 21 kDa were not observed for the unfermented substrates. This was likely at least partially because degradative enzymes were secreted by *A. oryzae* during fermentation. Only one protein bands was observed following fermentation with *A. oryzae* and *S. cerevisiae*. However, five protein bands (25, 28, 37, 43 and 50 kDa) were detected for substrates fermented with *A. oryzae* and *B. subtilis*, indicating fermentation increased the abundance of higher molecular weight proteins.

### Effect of SSF on In Vitro Digestibility

Data on in vitro digestibility of unfermented and fermented substrate is presented in Table 6. In vitro DMD, OMD, NDFD and ADFD before and after fermented were not significantly difference from each other, however, CPD was higher in the FFP than the unfermented substrate. The results of SDS-PAGE analysis also showed that SSF with mixed strains of *A. oryzae* and *B. subtilis* could increase the abundance of higher molecular weight proteins due to the various enzyme secreted by both strains, indicated that the FFP could be used as an alternative protein source in feed-stuffs in industrial applications by SSF since FFP exhibited the higher digestibility than the unfermented substrates.

**Table 6** In vitro digestibility of nutrients in the unfermented and fermented substrates for beef cattle

	Nutrients				
	DMD	OMD	CPD	NDFD	ADFD
Substrates					
US	57.4	52.2	81.9	23.7	17.6
FFP	55.1	53.8	87.9	22.8	19.1

But the feeding trials should be conducted in the future, for Israilides C et al. reported that feed efficiencies (g weight gain per g feed) and the acceptability were not significantly different between fermented sugar-beep pulp and the control [41].

### Conclusions

Fermentation of SPBR mixed with PS by *A. oryzae* and *B. subtilis* produced higher amounts of true protein than the other tested combinations. Under RSM-optimized conditions, the true protein content obtained was  $15.58 \pm 0.99\%$ , which was a 2–3 fold increase over the true protein content of unfermented substrates, the in vitro protein digestibility has improved after fermented but the feeding trials should be conducted in the future. The 10 kg-scale sweet potato beverage residues fermentation demonstrated a potential for treatment in industrial applications. Sweet potato beverage residues and peanut shells, which would otherwise be waste products, may be useful as solid substrates for protein enrichment by *A. oryzae* and *B. subtilis* during solid state fermentation.

**Acknowledgements** This study was financially supported by the international cooperation project “Utilization and preservation of agricultural by-products and crop residues for ruminant feeding in Israel and China” (2015DFG32360).

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