Bio-transformation of agri-food wastes by newly isolated *Neurospora crassa* and *Lactobacillus plantarum* for egg production

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ABSTRACT Using bio-transferred feedstuff was a cost-effective approach to improve egg quality and production; particularly, the nutritive diet came from agri-food wastes. In this study, optimization of fermentation conditions and co-cultivation of *Neurospora crassa* with *Lactobacillus plantarum* was performed in a simple bioreactor. The optimized fermentation of beer lees substrates through *N. crassa* led to the hydrolysis rates of crude fiber increasing to 43.27%. Compared to that of using *N. crassa* alone, the combination of *N. crassa* and *L. plantarum* enhanced the content of amino acids (13,120 to 18,032 mg/100 g) on oil-tea seed cake substrates particularly. When hens were fed 10% fermented oil-tea seedcake substrate, the ratio of feed to egg decreased from 3.1 to 2.6, egg production ratio increased from 65.71 to 80.10%, and color of vitelline (Roche) increased from 8.20 to 10.20. Fifteen kinds of carotenoids were identified by HPLC in fermented oil-tea seed cake substrates. The results of this study highlighted that the mixed-fermentation by *N. crassa* and *L. plantarum* may be an effective way to convert agri-food wastes into high-valued biomass products, which could have a positive effect on hens and their eggs.

Key words: *Neurospora crassa*, *Lactobacillus plantarum*, mixed-fermentation, agri-food wastes, egg production

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INTRODUCTION

Utilization of renewable lignocellulosic biomass, especially agri-food wastes, as substrates for fermentation can help alleviate serious environmental problems and waste of resources (Capel et al., 2011; Parrado et al., 2014). Most of these byproducts were recycled to produce ethanol or some bio-components through saccharification and fermentation. Nevertheless, the enrichment of nutrition in these agri-food wastes using biotechnology was overlooked. This could be an effective way of increasing their potential value as an additive for animal feed (Johnson et al., 2008). Of those techniques, there are 2 key issues restricting the application of recycling: the selection of experimental target substrates and the development of efficient bacterial strains for bio- transformation. In addition, the parameters of fermentation, the optimization of a nutritional proportion of raw materials, or even the subsequent animal tests need to be considered.

The bulk of wastes from agriculture or the food industry always consisted of a high ratio of cellulose and a certain amount of lignin; thus, they were not suitable for food or animal feedstuff. They were discarded, leading to an environmental problem (Liu et al., 2013). Oil-tea seed cake and beer lees were very common agri-food wastes in Southern China and can be obtained through the process of tea-seed oil and beer production. At present, these agri-food wastes are often used as fuel materials or fodder or employed for bioenergy production, e.g., ethanol, methane, etc. (Wan and Li, 2010). As mentioned above, the research on transformation of them into a nutritive stuff for livestock was quite limited. Actually, microbial pretreatment can enrich the nutrition content of biomass, e.g., Zhang found that fatty acids could be accumulated by mixed fermentation (Zhang et al., 2013). Hence, disposing these cellulosic biomasses through a bioprocess and improving their nutritional component ratio may provide an alternative option to tackle agri-food wastes. As shown in Fig 1, oil-tea seed cake and beer lees had a sufficient proportion of crude fiber and protein to fulfill the requirements of carbon and nitrogen sources for microorganisms.

Apart from these indexes, the constituents of cellu- lose and physical attributes benefited the growth of filamentous fungi (Suto and Tomita, 2001; Wan and Li, 2011), such as *Aspergillus niger* (Ramachandran et al., 2007) or even rumen microorganisms, in solid-state medium (Liu et al., 2013).

There were various studies of microorganism treatments in this field (Abdel-Rahman et al., 2013).
Fungus was considered to be the best choice of microorganism (Sreenath and Jeffries, 2011), e.g., production of thermophilic alkaline protease by *Streptomyces* sp. solid-state fermentation (SSF) (Lazim et al., 2009), the microbial conversion of recycled biomass resources to liquid and gaseous fuels (Hussain et al., 2011). Cellulolytic fungi such as *Trichoderma reesei* (Shin et al., 2000) and other fungi have been applied to the degradation of agricultural products, resulting in the accumulation of enzymes, soluble sugar, or other valuable products (Kim and Kim, 2012; Ma and Ruan, 2014), and then are utilized by functional microorganisms to promote the accumulation of bioactive substances that benefit health. For example, the monomeric sugars degraded from lignocellulosic biomass could transfer to lactic acid (Sreenath et al., 2001). However, the research on the application of bioconvertible materials, particularly on agri-food wastes, was inadequate and the interaction among multi-microorganisms in different solid-state substrates remained unclear. Therefore, it was necessary to verify the feed value of fermented biomass through animal tests.

Our laboratory isolated a kind of fungus, *Neurospora crassa* (*N. crassa*) CGMCC3088, which could secret cellulases to decompose fiber to oligosaccharide. We have confirmed that the fungus was one of the mutants of *N. crassa*, a well-studied fungus used in genetics and protease studies (Yazdi et al., 1990; Kritskii et al., 2001). *N. crassa* was treated as a functional fermentation organism, for it can yield carotenoid and optimize nutritional components of substrates through its endogenous enzymatic systems (Yoshida et al., 2006). In addition, *N. crassa* is a kind of epiphyte that possesses the capability of reproducing hypha-secreting cellulases. It, however, is an anaerobe. *L. plantarum* is a facultative anaerobe and can breed even at the bottom of substrates where oxygen is limited. Apart from these, *L. plantarum* is a typical probiotics belonging to gut microorganisms, which is able to produce lactic acid and other probiotic substances to improve the microenvironment of the intestinal tract (Ge et al., 2009).

This inspired the idea of combining these 2 microbes so that the diet fiber in agri-food wastes could be degraded into oligosaccharide to improve prebiotic growth, nutritional content, and bio-efficiency for animals.

The objective of this study was to investigate the feasibility of fermentation with *N. crassa* alone as well as combined with *L. plantarum* to bio-transform worthless agri-food wastes to fermented animal feeds. Therefore, we compared the nutrients using oil-tea seed cake and beer lees as grown substrates and selected the suitable substrate for feeding experiments in hens, determining the effect of bioconverted agri-food wastes on feed. For this purpose, feeding efficiency and egg quality were determined by adding mixed fermented oil-tea seed cake substrate as one component instead of the common contents in the hens' feed recipe.

**MATERIALS AND METHODS**

**Materials**

The beer lees (crude fiber 17.51%, protein 33.49%, soluble sugar 11.12%) were obtained from Wanan Brewery, Jiangxi Province, China. Oil-tea seed cake (crude fiber 26.88%, protein 9.90%, soluble sugar 8.15%) was obtained from Qinglong High-Tech Company (Yichun County, Jiangxi Province), and only 1.13% of tea saponin remained with 70% of ethanol and ultrasonic extraction. All these raw materials were dried in an oven at 50°C to a constant weight and were pulverized to the particle size of 0.42 mm. All other reagents used in this study were of analytical grade.

**Cultures**

A strain of *N. crassa* CGMCC 3088 used in the study was previously isolated from Tofu (Ruijin, Jiangxi Province, China). The strain was characterized, identified by Guangdong Culture Collection Center, and deposited at State Key Laboratory of Food Science
Table 1. Experimental design and results of CCD of response surface methodology for the optimization of degradation ratio of crude fiber.

<table>
<thead>
<tr>
<th>Run number</th>
<th>Initial pH</th>
<th>Fermentation temperature (°C)</th>
<th>Fermentation period (h)</th>
<th>Degradation ratio of crude fiber (%)</th>
<th>Beer lees substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−1</td>
<td>−1</td>
<td>0</td>
<td>40.74</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>−1</td>
<td>1</td>
<td>0</td>
<td>32.80</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>−1</td>
<td>0</td>
<td>33.07</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>39.34</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>−1</td>
<td>−1</td>
<td>47.17</td>
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</tr>
<tr>
<td>8</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>45.63</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>−1</td>
<td>0</td>
<td>−1</td>
<td>32.94</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td>−1</td>
<td>40.83</td>
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<tr>
<td>11</td>
<td>−1</td>
<td>0</td>
<td>1</td>
<td>47.70</td>
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<td>43.68</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0</td>
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<td>0</td>
<td>47.57</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>47.56</td>
<td></td>
</tr>
</tbody>
</table>

and Technology Nanchang University, China. The fungus was aseptically incubated on potato dextrose agar (PDA) for 4 d at 37°C, stored at 4°C in sterile conditions, and subcultured once a month.

*L. plantarum* NCU116 strain was isolated by our research group from local pickled vegetables and verified through molecular biology identification (Li et al., 2014). Anaerobic liquid fermentation of the strain with Man Rogosa Sharpe (MRS) liquid substrates was carried out in a bioreactor for 24 h at 25°C and then stored on slopes of 5% (v/v) solid malt medium at 4°C in State Key Laboratory of Food Science and Technology, Nanchang University, China.

**Inoculum Preparation**

To prepare inoculum of *N. crassa* for SSF, 10 mL Tween-80 (0.1%, v/v) was added to fully sporulated (7-day-old) PDA agar slope cultures. The spores were scraped off under aseptic conditions with sterile distilled water to produce a spore suspension to be used as the inoculum (1 × 10⁷ spores/ml). For inoculation, *L. plantarum* strains were streaked on the MRS agar plate and incubated at 37°C for 48 h as the seed liquid for SSF.

**Optimization of Fermentation Conditions for Beer Lees**

The fermentation condition for the substrates of oil-tea seed cake was the same as a previous study (Xiao et al., 2010). The optimal fermentation conditions of oil-tea seed cake were optimized at the ratio of solid medium to distilled water of 1:2.6, initial pH 7.0, fermentation time of 74.9 h, and inoculum level at 2.5% (v/m).

The fermentation conditions for the substrates of beer lees were optimized through the one-factor-at-a-time method and response surface methodology (RSM) in this assay. There was a total of six variables (sterilization time, initial pH, the rate of solid medium to distilled water, incubation temperature, fermentation period, and inoculum level) in the one-factor-at-a-time experiments (data not shown). Based on these results, 3 optimal parameters (initial pH, fermentation temperature, and fermentation period) were obtained with the RSM using the central composite design (CCD). Each design of variables used in RSM was at 3 different levels, and all experiments were taken at a central coded value of zero (Supplementary Table 1). Experimental design and results of RSM for the optimization of degradation ratio of crude fiber are shown in Table 1.

**Mixed Fermentation by *N. crassa* and *L. plantarum***

The optimized fermentation conditions of beer lees substrates and oil-tea seed cake substrates were also performed on mixed-fermentation. The inoculum proportion of *N. crassa* or *L. plantarum* on beer lees substrate and oil-tea seed cake substrate was modified through the one-factor-at-a-time classical method for mixed fermentation (Supplementary Fig 1). The total inoculum levels of strains on both substrates were in accordance with previous conditions.

**Culture Media of Agri-Food Wastes**

The culture media of oil-tea seed cake and beer lees were optimized with response surface methodology analysis in our previous studies. The oil-tea seed cake substrate consisted of oil-tea seed 52.48%, bean dregs 29.38%, and rice bran 18.54%, according to previous studies (Xiao et al., 2010). The beer lees substrate was made up of beer lees 49.08%, bean dregs 26.08%, (NH₄)₂SO₄ 3.71%, KH₂PO₄ 1.53%, CaCl₂ 0.24%, and MgSO₄ 0.08% (Jia et al., 2009).
**Animal Trial**

In order to determine the feasibility of the fermented products to be used as feedstuff, the effect on egg production as well as metabolic rate of hens were evaluated.

**Animals and Diets**

Sixty healthy HaiLan hens (aged 400 d, body weight approx. 1.69 kg) were obtained from Hongmen Enterprise Co. Ltd. (Nancheng County, Jiangxi Province, China) and divided into 4 groups randomly: group 1 (control group without additional fermented material); groups 2 to 4 (containing 5, 10, and 15% fermented material, respectively). Every group had 5 replications with 3 hens in one cage. The animals were kept in the henhouse of the laying hen farm, Jinagxi Province, China, and allowed to acclimate for one wk. Before the experiment, hens were fasted for 12 h in order to discharge the contents in the alimentary canal. The specific formula of feedstuff and calculated nutrient contents in the original recipe of feedstuff, which was used as the control.

**Feeding Efficiency of Fermented Oil-Tea Seed Cake Substrate**

The body weights of all hens were measured weekly. Eggs and remaining diet were collected every d to calculate the laying rate and the ratio of feed to egg. Laying rate was the average number of eggs produced from every hen divided by the number of experimental d. Egg quality was determined by several indexes (Table 3). For example, the index of egg shape from 1.3 to 1.35 was considered normal shape. The index of Haugh unit indicated the quality and freshness degree of the eggs. The higher number of this index, the better quality the eggs had. These indexes were measured by the egg quality analysis meter (EMT-5200 Robotmation Japan). The rate broken of eggs represented the solidity of eggshell, which was the ratio of damaged eggs to total eggs.

Hens were fed at 9:00 am and 4:00 pm, under 16 h light/8 h dark and well-ventilated conditions. During the last 5 d of the experiment, all feces from each group were collected at 8 am in order to measure the metabolic rate of nutrients. One hundred g feces were mixed with 10 mL 10% hydrochloric acid, dried at 70°C immediately, and then the crushed samples were sealed for storage. The total daily feed intake = the amount of daily added feed – the amount of daily remaining feed. The apparent metabolic rate of dry substance, crude protein, and ash for each diet was determined using the following formula: apparent metabolic rate (%) = [(the intake – the output)/intake] × 100.

**Content and Category of Carotenoids on Mixed Fermented Oil-Tea Seed Cake**

It was supposed that the color variation of yolk may be attributed to the production of carotenoids from *N. crassa*, so the content and categorization of carotenoids on fermented substrate was carried out. Total carotenoids quantification was performed with UV-vis spectrophotometer (Sp-1910UCPC, Shanghai Spectrum Instruments Co. LTD). The carotenoids were extracted with pure acetone for one h using an acid-heat method. After incubation, the fermented media were dried at 50°C until a constant weight, then sieved for the following extraction procedure. One gram of substrate was loaded in 30 mL centrifuge tube and soaked in one mol/L HCl with a solid-to-liquid ratio of 1:5 for 5 min. The solid-liquid mixture was dried with vacuum suction filtration. The obtained solids were added to 40 mL of acetone to extract the carotenoids for one h, followed by centrifugation (4,200 rpm, 10 min). The absorbance of carotenoid extract was determined by UV-vis spectrophotometer at 461 nm. The content of total carotenoids was calculated according to the following formula Eq. (1) (Papaoannou and Liakopoulou-Kyriakides, 2012):

\[
CY = A \times D \times V/0.16/w
\]

Where CY indicates the carotenoids yield (μg/g dry substrate), A is the absorbance of diluted extract solution at 461 nm, D is the dilution ratio, V is the volume of acetone added, 0.16 is the extraction coefficient of carotenoids, and W (g) is the weight of the dry substrate. Carotenoids yield was expressed as μg/g dried substrate.

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**Table 2. Formulation of feed dietary and nutrients levels.**

<table>
<thead>
<tr>
<th>Content</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermented substrate (%)</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Corn (% , 15.6% CP)</td>
<td>59.20</td>
<td>57.80</td>
<td>55.20</td>
<td>52.70</td>
</tr>
<tr>
<td>Bean pulp (% , 23% CP)</td>
<td>25.00</td>
<td>23.20</td>
<td>20.80</td>
<td>18.30</td>
</tr>
<tr>
<td>Bran (% , 52% CP)</td>
<td>2.00</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fish powder (% , 62.9% CP)</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Calcium %</td>
<td>8.00</td>
<td>8.00</td>
<td>8.00</td>
<td>8.00</td>
</tr>
<tr>
<td>Calcium hydrophosphate 2</td>
<td>1.50</td>
<td>1.50</td>
<td>1.20</td>
<td>1.00</td>
</tr>
<tr>
<td>Salt (%)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Additive (minerals and vitamins)</td>
<td>0.80</td>
<td>0.80</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>Calculated nutrient content</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat (%)</td>
<td>1.00</td>
<td>1.20</td>
<td>1.50</td>
<td>1.70</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>17.37</td>
<td>17.19</td>
<td>16.93</td>
<td>16.64</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>4.12</td>
<td>4.10</td>
<td>4.07</td>
<td>3.98</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>0.3</td>
<td>0.31</td>
<td>0.31</td>
<td>0.32</td>
</tr>
<tr>
<td>ME(Mcal/kg)</td>
<td>2.83</td>
<td>2.80</td>
<td>2.76</td>
<td>2.73</td>
</tr>
<tr>
<td>Protein/ME(g/Mcal)</td>
<td>61.38</td>
<td>61.39</td>
<td>61.34</td>
<td>61.40</td>
</tr>
</tbody>
</table>

Note. 1Sigma Chemical Co. with a purity of 98%. 2Supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 6,000 IU; vitamin D3, 3,000 IU; vitamin E (DL-α-tocopheryl acetate), 25 IU; menadione, 1.5 mg; thiamine, 1.5 mg; riboflavin, 1.5 mg; niacin, 10 mg; pyridoxine, 1.5 mg; vitamin B12, 0.80 μg; folic acid, 0.5 mg; biotin, 600 μg; iron, 80 mg; zinc, 60 mg; manganese, 50 mg; copper, 10 mg; iodine 0.8 mg; and selenium, 0.3 mg.
The carotenoid extract was concentrated under nitrogen gas and then re-dissolved in one mL of a mixture of methanol/acetonitrile/isopropanol alcohol, 54:44:2, V/V). HPLC was carried out using an Agilent 1100 series system equipped with a G1379A degasser, a G1311A quat pump, a G1357D high-performance autosampler (HIP ALS SL+), and a G1316B SL thermostatted column compartment. Separation of the carotenoids was achieved on a Hypersil ODS C18 column (5 μm particle size 250 mm × 4.6 mm id, Thermo Electron Corporation, Waltham, Massachusetts) with methanol/acetonitrile/isopropanol alcohol (54:44:2, by volume) as the mobile phase for isocratic elution at a flow rate of 0.8 mL/min, and the column temperature was 30°C. The peaks were identified by their retention time and respective spectra recorded with the DAD photodiode array detector, compared with either standards or with spectral data previously reported.

**Measurement of Nutritional Components**

Crude fiber was determined by the method described on the AOAC Official Method 962.09. Protein amount was measured using Kjeldahl method described on AOAC Official Method 990.02 Determination of soluble sugar was carried out according to AOAC Official Method 945.29. Free amino acid was measured by AOAC Official Method 985.28.

**Statistical Analysis**

Results in triplicates were expressed as means ±SD. Statistical analyses were performed by SPSS13.0 for windows (SPSS Inc, Chicago, IL). Data assembled to evaluate nutritional differences between groups were determined by Random Effect Model and ANOVA followed by Tukey’s test. Values in the same row with different letters show significant differences (*P < 0.05).

In this experiment, P-value for regression analysis is 0.04.

Data not sharing a common superscript letter (a-c) in same row are significantly different, (*P < 0.05).

The effect of fermentation conditions for the optimum degradation of crude fiber in beer lees was performed by RSM. Based on the results of the one-factor-at-a-time method and the plan of Plackett-Burman design, 3 critical factors (initial pH, fermentation temperature, and fermentation period) were selected to determine the optimum condition of SSF of N. crassa. The coefficients and significance of the response surface model were assessed by regression analysis. The coefficient of determination (R²) of beer lees substrates was 0.9926, (P = 0.0422). These results suggested that the model represented the real interaction among the parameters tested. The predictive equation acquired was as follows: (A is initial pH, B is fermentation temperature, C is fermentation period)

\[
\text{Degradation ratio of crude fiber in beer lees} = 46.27 - 0.13A - 0.5275B + 1.9175C - 5.2388A^2 + 3.5525AB + 3.4125AC - 4.5438B^2 + 5.4075BC - 0.7338C^2
\]

Through the verification experiment, the optimum fermentation conditions of beer lees were determined as follows: sterilization time 20 min, initial pH 5.4, ratio of solid medium to distilled water 1:3.5.
incubation temperature 28.5°C, fermentation period 56.7 h, seed age 42 h, and inoculum level 10% (v/m). The factor interactions (AB, AC, and BC) and square of the parameters (A², B², and C²) used in this study were a marked difference. The degradation rate of crude fiber in beer lees substrate under the optimized conditions through RSM was found to be 47.7%, which manifested the significant difference (P < 0.05 Table 1).

**Effect of N. crassa on Nutrient Improvements in Optimized Substrates**

SSF was responsible for the production of secondary metabolite by microbes (Niladevi et al., 2007). As shown in Fig 1, the levels of protein and total soluble sugar of beer lees and oil-tea seed cake substrates increased significantly after fermentation by N. crassa (P < 0.05). In particular, the soluble sugar content of oil-tea seed cake increased by 373.29% (P < 0.01), while the beer lees contained a high level of protein (34.82%). In contrast, crude fiber levels in these 2 substrates significantly declined, especially for oil-tea seed cake, as the degradation ratio reached 53.90% (P < 0.05). The high efficiency on the reduction of crude fiber proved that N. crassa exhibited the ability to degrade crude fiber and live on decomposed low-value materials. This may be mainly because the optimized culture substrates and fermentation conditions boosted the production or activities of cellulases (de Castro et al., 2010; Vishwanatha et al., 2010). Our research group separated exo-1,4-β-D-glucanases (CBH), endo-1,4-β-D-glucanases (EG), and β-Glucosidases (CB) from N. crassa CGMCC 3088. EG and CB were reported to have relatively high activities in this enzyme complex. SSF facilitated the penetration of mycelia and expanded the contact surface area on different cellulose structures, which was helpful for the yield of cellulases from fungus (Thygesen et al., 2011). Apart from fermentation conditions, the category of substrate could play a part in the production of cellulases, thus leading to the difference in degradation ratio of crude fiber. It was found that the substrate composition discrepancy had an influence on enzyme production (Yamane et al., 2002).

The initial total content of amino acids of beer lees and oil-tea seed cake were 6,324 mg/100 g and 6,162 mg/100 g (wet weight), respectively, and increased dramatically to 9,886 mg/100 g and 13,129 mg/100 g for corresponding fermented substrates. It was noticed that increments on fermented substrates of oil-tea seed cake were much higher than those of the beer lees substrates, especially for glutamic acid, valine, and essential amino acids. The major amino acids presented in the fermented samples were aspartic acid and glutamic acid. A heat map showed the relative abundance of different amino acids before and after fermentation (Fig 2). The results suggested that boosted soluble sugar contributed to simultaneous crude fiber hydrolysis and the saccharification process.

**Synergy of N. crassa and L. plantarum on Nutrient Improvements in Optimized Beer Lees and Oil-Tea Seed Cake Substrates**

Enhancement of the nutritional component of agricultural products was commonly considered as a major benefit of mixed fermentation (Mishra et al., 2013; Ma and Ruan, 2014). According to the one-factor-at-a-time method (Supplementary Fig 1), the optimal proportion of N. crassa and L. plantarum inoculated into beer lees substrate was 5% (v/m) and 5% (v/m), and in the oil-tea seed cake substrate 4% (v/m) and 6% (v/m). The total inoculum was in accordance with the previously mentioned. Compared with the raw substrates, the levels of protein increased from 28.53 to 36.74% and 13.94 to 21.32% (P < 0.05), while that of soluble sugar increased from 8.74 to 16.41% and 7.6 to 23.18% in mixed-fermented beer lees substrate and mixed-fermented oil-tea seed cake substrate, respectively (Fig. 1 & Supplementary Table 2 ).

In contrast to mono fermentation, the protein level of mixed fermented beer lees substrate and oil-tea seed cake substrate increased by 5.51 and 9.67%, respectively. This may result from cellular protein of L. plantarum and high protein transfer ratio. The content of soluble sugar declined sharply to 16.41% and 23.18% for beer lees and oil-tea seed cake substrate, respectively, and the reduction ratio was 48 and 35.56%, respectively (Fig. 1). The efficiency of fiber degradation was similar to mono fermentation on beer lees substrate; however, the results on oil-tea seed cake substrate was relatively promising, and the crude fiber level descended to 10.38%. Apart from the above mentioned, we
particularly pointed out that every kind of amino acids on mixed fermentation of oil-tea seed cake substrate had an ascent compared with mono fermentation, except alanine, which declined from 779 mg/100 g to 89 mg/100 g. The content of aspartic acid, glutamic acid, leucine, and tyrosine increased significantly to 2,742 mg/100 g, 3,437 mg/100 g, 1,757 mg/100 g, and 1,011 mg/100 g, respectively; the total quantity of amino acid reached 18,032 mg/100 g (Fig. 2).

These data indicated that better results can be obtained using mixed fermentation by both N. crassa and \textit{L. plantarum} than by \textit{N. crassa} alone. This might be because the enzymes produced by \textit{N. crassa} could degrade lignocellulosic material and transform them into cellobiose, glucose, and some kinds of fermentable sugar. As a consequence, \textit{L. plantarum}, a heterotrophic bacterium, could make the most use of fermentable soluble sugar as an energy supplier to synthesize proteins and enrich the content of amino acids (Ozmihci and Kargi, 2010). Correlation analysis showed that the soluble sugar decline was proportional to the inoculum level of \textit{L. plantarum}. Apart from these, the protein content with the total amount of amino acids concurrently increased, thus proving the aforementioned hypothesis.

In order to investigate the interaction between \textit{L. plantarum} strain and \textit{N. crassa} on culture environment, MRS state plating medium was used to culture \textit{L. plantarum} and \textit{N. crassa}, with bromcresol purple as an indicator. In an anaerobic environment, there was only the activity of \textit{L. plantarum} that yielded a lot of acidic substances. The color of the substrate changed from purple to yellow, and the pH value declined from 6.5 to 4.0. The culture medium was then put in an aerobic environment, and the color changed back to purple and the pH value went back to around 7.0. This may be because \textit{N. crassa} took advantage of the organic acids produced by \textit{L. plantarum} as a carbon source, which led to the change in pH. In addition, it was observed that the addition of an \textit{L. plantarum} strain to \textit{N. crassa} significantly improved the ability of fungus to grow on the agri-food wastes, and made the hypha adhere to substrates more stably and tighter. It could even be found that the hypha penetrated the solid substrates to the bottom (Supplementary Figure 2). SSF was previously described as being more available for mixed strains, by resembling the natural environment, and can work synergistically in bioconversions (Delabona Pda et al., 2012; Kolasa et al., 2014). These results demonstrated that the fungal-lactobacillus interaction in co-cultivation significantly enhanced utilization of agri-food wastes, while increasing the production of proteins, soluble sugar, and amino acids with respect to raw wastes. In addition, possible toxic substances, such as deoxynivalenol, zearalenone, and aflatoxin B1, were not detected in the fermented substrates from \textit{N. crassa} and \textit{L. plantarum}.

**Feeding Efficiency of Oil-Tea Seed Cake Substrates with Fermentation by 2 Mixed Microbes for Hens**

As one of the most abundant nutrients, bioconverted materials are able to be added to feedstuff as an ingredient substitution (Biesebeke and Record, 2008). Beer lees have been fully utilized in the recycling industry (White et al., 2008; Khattak et al., 2013), especially after microbiological treatment, and there is an obvious increase in cellular protein (Aggelopoulos et al., 2013). This study was the first trial to make oil-tea seed cake a component of feedstuff. In this work, we discovered the fermented oil-tea seed cake substrate had more improved nutrients than beer lees and contained natural antioxidant compounds, so it was expected to be an additive of feedstuff for hens to enhance egg production and quality.

The results of the animal trial are shown in Table 3. For group 2 (5%) and group 3 (10%), the laying rate reached 80%, and the amplitude was 23.19 and 21.74%, respectively. Groups 2 and 3 had the highest efficient conversion of feed to egg (2.4 to 2.6), but possessed similar data on average weights of eggs. Group 3 (10%) possessed the highest metabolic rate of dried substance, crude protein, and ash. However, group 4 (15%) had the lowest laying rate and metabolic rate of dried substance, crude protein, and ash. This means that 15% fermented substitute is unable to fulfill the energy requirement, which leads to the decline of metabolic rate. The Haugh unit, color of vitelline, and index of egg shape in the test groups were better than that of the control group. The Haugh unit indicates the viscosity of albumen, which was mainly determined by the concentration of thick albumen. The thicker the albumen, the more viscosity the egg had, as well as more extended freshness. The age of the laying hens and storing conditions were the main factors contributing to the content of thick albumen. With the aging of hens, the content of thickness went down and the Haugh unit declined accordingly. When the unit is below 65, there is no market for this kind of egg. Groups 3 and 4 showed the best data on the Haugh unit, indicating the more fermented ingredients were involved, the better the performance of egg quality and maintained freshness. The color of vitelline in group 4 was better than those of groups 2 and 3 (P < 0.05). Addressing the satisfactory phenomenon on vitelline color, we considered whether carotenoid yielded by \textit{N. crassa} may play a positive role. There were reports on the bio-transformation of feed supplements for enrichment of the active substances in yolk. Feeding oil byproducts to laying hens led to an accumulation of conjugated linoleic acid in egg yolk (Kim et al., 2008). We tested the category and content of carotenoids in the mixed fermented oil-tea seed cake substrate. The HPLC chromatography of the carotenoids produced by \textit{N. crassa} is shown in Fig. 3. The probable compounds corresponding to the
peaks are shown in Table 4 (Arrach et al., 2002; Saelices et al., 2007). The results showed that the total content of carotenoids was 1,012 μg/gds, and 15 kinds of carotenoids were identified by HPLC. The strong intensity of the absorption peak of neurosporaxanthin, torulene, and all-trans-β-carotene indicated that these 3 types were the main components of carotenoids produced by N. crassa. The ratio of chorion, vitelline, and albumen was measured in boiled eggs, which had filmy chorion and more vitelline than albumen in group 3.

According to the results mentioned above, groups 2 and 3 exhibited comprehensive benefits on hen egg number and hens’ conditions due to the addition of fermented feedstuff. This could be explained by the following aspects. First, the initial formula composited higher energy but the actual availability of it was not as good as the fermented product because the undigested crude fiber inhibited absorption of some nutrients in the hens’ intestines; nevertheless, N. crassa could degrade crude fiber into cellobiose, glucose, and some kinds of soluble sugar, which were absorbed and may reinforce the growth of probiotics in the gastrointestinal (Donalson et al., 2008). Second, oil-tea seed cake originally contains oleic acid and tea polyphenols, which are able to enhance the antioxidant ability of hens, and the incremental free amino acid can be absorbed smoothly without characteristic enzymolysis. Third, L. plantarum broadens the cellular protein and category of amino acid, and its metabolites, such as lactic acid and exopolysaccharide, could improve the intestine
environment of the test animals and then improve intestine health (Abdel-Rahman et al., 2013). Finally, *N. crassa* synthesized an abundant amount of carotenoids during the fermentation process, which hens absorbed and accumulated in the yolk, improving the color of vitelline and raising the value of the eggs. All of the above mentioned aims to improve the hens’ health and lead to high egg production and quality. Furthermore, the less costly fermented oil-tea seed cake substrate of *N. crassa* and *L. plantarum* substituted some high cost ingredients in the recipe of laying hens’ feed and has been proven to enhance feeding efficiency and egg production.

**CONCLUSION**

These assays proved the availability of *N. crassa* CMCC3088 for eliminating crude fiber and improving the nutritional characteristics of agri-food wastes. Compared to other microorganisms that produce only cellulases, *N. crassa* might be a potential better candidate for extending the value of agri-food wastes by its stronger producing ability in nutritive substrates. The combination of *N. crassa* and *L. plantarum* showed compatible fermentation with high efficiency of nutrient transformation. The bioconverted oil-tea seed cake substrate used as an additive to feedstuff was unexpectedly proven to enhance feeding efficiency and egg production. This research provided an option for agri-food wastes bioconversion to poultry feeds, which can be a potentially cost-saving factor for the poultry industry.

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**COMPETING INTERESTS**

The authors declare that they have no competing interests.

**SUPPLEMENTARY DATA**

**Supplementary Table S1.** Central composite design level.

**Supplementary Table S2.** Nutrient component variations in oil-tea seed cake substrate.

**Supplementary Figure S1.** Effect of inoculum proportion on mixed fermentation. The horizontal axis stands for the percentage of *N. crassa*’s inoculum and the total inoculum is 10%. The remaining inoculum proportion is from *L. plantarum*.

**Supplementary Figure S2.** Comparison of mono- mixed growth condition on oil-tea seed cake.

**Supplementary data is available at PSA Journal online.**

**REFERENCES**


