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Zhe Yang
Shandong University of Technology
Min Zhang
Shandong University of Technology
Lijun Jiang
Shandong University of Technology
Wenjing Suo
Shandong University of Technology
Yuxin Deng
Shandong University of Technology
Haijing Zhang
Shandong University of Technology
Peng Guo
Shandong University of Technology
Hongjun Li (✉ Hongjunli1351@hotmail.com)
Shandong University of Technology

Research Article

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Posted Date: June 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-609924/v1

Optimization of Medium for Bioconversion Extruded Apple Pomace into Microbial Protein

Zhe Yang, Min Zhang, Lijun Jiang, Wenjing Suo, Yuxin Deng, Haijing Zhang, Peng Guo, Hongjun Li*

School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, Shandong, China

*Corresponding Author:
Hongjun Li,
School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, Shandong, China;
Email: Hongjunli1351@hotmail.com

Abstract: The medium compositions such as carbon and nitrogen sources, moisture content and inorganic salt affected the microbial protein (MP) production. Imbalance of carbon-nitrogen ratio in apple pomace (AP) limited the microbial utilization. Hence, those conditions must be optimized to achieve maximum MP. In this work, AP was pretreated by extrusion technology to obtain extruded apple pomace (EAP). Subsequently, the medium compositions were optimized using Plackett-Burman design (PBD) and Box-Behnken design (BBD). PBD determined four significant factors (bran, glucose, packing quantity (PQ), water to material ratio (W/M)) out of the eight variables. The BBD results showed that optimal true protein content (10.42%), effective viable count (1.94×10^9 CFU/g) and crude protein content (18.73%) were achieved at bran 16.22%, glucose 8.09%, PQ 9.88 g and W/M 1.56. Compared with AP, the true protein and crude protein content of optimal fermented EAP (FEAP) were increased by 152% and 216%, respectively. According to fluorescence microscopy, the cellulose of AP was little effected by extrusion technology while was mostly degraded by mixed strains (*Aspergillus niger*, *Candida utilis*, *Geotrichum candidum* and *Lactic acid bacteria*). Combination of extrusion and fermentation, the medium compositions were optimized to promote the bioconversion of AP into MP feed.

Keywords: Extruded apple pomace; Medium compositions; Mixed strains Fermentation; Plackett-Burman design; Box-Behnken design

Introduction

Apple pomace (AP) is the primary by-product generated in manufacturing apple juice and accounts for 30% of the weight of processed apples (18). Most of AP were discarded as waste (10), causing environmental pollution and waste of resources. With a small amount used to produce organic acids, aroma compounds, bioethanol, enzymes among others (9, 11, 24). However, its application on industrial scale was limited due to high production cost (23). Several studies shown that AP was converted into high quality microbial protein feed which effectively improved its nutritional value (28, 29). Microbial protein (MP) could mitigate the stress on the food chain and environment by providing a more sustainable, high quality protein source (26). The composition of AP indicated the presence of significant quantity of insoluble carbohydrates, small amounts of proteins and essential amino acids (8). Its imbalance of carbon-nitrogen ratio limited microbial utilization.

Extrusion technology showed great potential for large-scale production with various advantages: high shearing force, easy to operate, and excellent temperature control (15). Extrusion pretreatment would not induce the formation of fermentation inhibitors (14), and it also had little effect on the content of basic compositions of AP. In our previous work, the protein content obtained by extruded apple pomace (EAP) fermentation needed to be further improved. Therefore, the fermentation substrate should be added various nutrition substances to make it more suitable for microbial growth.

The medium compositions that affected the fermentation process mainly included carbon sources, nitrogen sources, moisture content, inorganic salt and so on. In recent years, Campbell et al. (6) suggested that the digestibility of crude protein, total amino acids of the fermented sweet potato were improved with the addition of 1 g KH_2PO_4, 0.5 g $MgSO_4$, and 0.5 g $MnSO_4$ and 0.5 g $ZnSO_4$ per liter. Rodríguez-Muela et al (25) reported that 1.5% urea and 0.4% $(NH_4)_2SO_4$ were added in the process of bioconversion of apple pomace. Thus, the design of appropriate fermentation medium was extremely important for the optimization of fermentation products.

Plackett-Bruman design (PBD) is two-level fractional design for studying up to k=N-1, where k is the number of variables, and N is the number of runs. This design was generally preferred for screening of significant factors (22). Response surface methodology (RSM) has been an effective statistical technique for the investigation of complex processes. RSM consists of a group of mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables. RSM mainly contains Central Composite Design (CCD) and Box-Behnken design (BBD), BBD includes all global designs and requires only three levels of factors to be operated, can be rotated completely or partially. It usually had concerned optimization of the composition of growth and production culture medium (20). Tang et al (27) reported that the fermentation substrate compositions were optimized for producing xylosidase from corn cob fermented by *Aspergillus niger*, using Plackett-Burman experiment, path of steepest ascent experiment and RSM. Statistical experimental designs such as PBD and RSM have been successfully applied to optimize many bioprocesses (1-3).

To our knowledge, there are few reports on the optimization of medium for EAP fermentation to produce MP feed. In this study, true protein (protein nitrogen, which obtained after separation of the non-protein nitrogen fraction from total nitrogen) (7), crude protein, and effective viable count were used as indicators, and EAP was used as raw material, to optimize the medium compositions for converting EAP into MP-rich feed.

Materials and methods

Materials

AP was provided by Kangyuan Biotechnology Co. Ltd (Zibo, China). Bran was supplied by Zibo Flour Mill (Zibo, China). Peptone, yeast extract, agar, and Man Rogosa Sharpe (MRS) medium were obtained from AOBOX (Beijing, China). Chemicals including glucose, urea, etc. were obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Distilled water was prepared in the laboratory. *Aspergillus niger* 3.324 (*A. niger*) and *Lactic acid bacteria* (LAB) were preserved at Laboratory of School of School of Agricultural Engineering and Food Science, Shandong University of Technology (Zibo, Shandong). *Candida utilis* 1314 (*C. utilis*) and *Geotrichum candidum* 1315 (*G. candidum*) were purchased from China Center of Industrial Culture Collection (Beijing, China).

Extrusion pretreatment

AP was pretreated by using single screw extruder (School of Agricultural Engineering and Food Science, Shandong University of Technology) with the extrusion parameters of screw speed 160 rpm, sleeve temperature 110℃ and material moisture content 26%. EAP was dried in an oven at 60℃ to constant weight, ground by the ultrafine crusher and filtered through 60 mesh screen.

Fermentation

A. niger was cultured on Martin MediuM Modified which contained 0.5% peptone, 0.2% yeast extract, 2% glucose, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$. *C. utilis* and *G. candidum* were cultured on Yeast Extract Peptone Dextrose (YPD) Medium containing 1% peptone, 0.5% yeast extract, 2% glucose at pH of 7.0~7.2. LAB was cultured on Man Rogosa Sharpe (MRS) Medium. *A. niger*, *C. utilis* and *G. candidum* were incubated at 30℃and 160 rpm for 24h, and LAB at 37℃ and 160 rpm for 24 h.

Fermented EAP (FEAP) was obtained that EAP inoculated with 10% (v/w) mixed strains suspension at 30℃ for fermentation 96 h.

Index determination methods

True protein content (TP) was determined following as: the true protein of sample was precipitated by salting out with $CuSO_4$ under the alkaline condition. Then, the precipitation was determined by K9860 automatic kjeldahl nitrogen meter (Shandong Haineng Scientific Instrument Co., Ltd, China). Crude protein content (CP) was determined by K9860 automatic kjeldahl nitrogen meter.

Effective viable count (EV) was analyzed following as: sample (1.00 g) was suspended in 9 mL of sterile distilled water by using the vortex mixer for 10 min. Then the suspension was gradient diluted in sterile distilled water and inoculated onto agar plates of Rose Bengal Medium (5 g peptone, 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.033 g rose Bengal, 0.1 g chloramphenicol and 20 g agar per liter) and MRS Medium. Triplicate plates were prepared for the colony counting of each sample, and were incubated for colony growth at 30℃ for 48 h.

Experimental design

Plackett-Burman Design (PBD)

The Plackett-Burman design is an efficient screening method to identify the significant medium compositions that influence the fermentation process (5). In this work, 10% (v/w) strain suspension was inoculated in each 200 mL Erlenmeyer flask containing sterilized substrate, those flasks were incubated at 30℃ for 4 days. Eight individual variables including urea, $(NH_4)_2SO_4$, bran, glucose, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, packing quantity (PQ), ratio of water to material (W/M) were investigated in order to determine significant variables effects on TP (Y_1), EV (Y_2) and CP (Y_3) of FEAP. The details of variables and there levels were showed in Table 1.The variables which showed significant effect ($p<0.05$) on responses, were considered for further optimization by RSM.

Path of steepest ascent method

The optimum level of each selected variable based on the results of the PBD was examined by the path of steepest ascent method (30). According to the positive and negative effects of significant factors, and a reasonable step length was designed to approach the region with the best effect. The optimal medium compositions obtained from this experiment were used as the center-points for RSM.

Box-Behnken Design (BBD)

Four significant factors for MP feed production had been obtained by PBD. Each of the independent variables was studied at three levels (−1, 0, 1). The details of variables and there levels were showed in Table 2. The quadratic polynomial equation was used to calculate the relationship between the independent variables.

Fluorescence microscopy

AP, EAP and FEAP (fermented product obtained by fermentation under optimized medium conditions) were stained with a few drops of fluorescent Congo red dye (0.2%, w/v) at room temperature 30 min. The stained samples were put on a glass slide and covered with a coverslip (17). The observation was conducted using Nikon 50i fluorescence microscope.

Statistical analysis

The data were analyzed by Design-Expert 8.0.6 and Minitab 19. Origin 9.0 was used to plot the data. All the experiments were expressed as the means ± Standard Deviation (SD).

Results

Screening of variables with significant influence on FEAP

A total of eight variables had been investigated with respect to their effect on TP, EV and CP of FEAP using PBD. The design of experiments and the corresponding responses were shown in Table 3. The variation suggested that the optimization process was important for improving the nutrition value of EAP. The following model equation had been respectively obtained for TP (Y_1), EV (Y_2) and CP (Y_3) (Eq. 1, 2 & 3)

$$Y_1=8.04+0.029X_1-0.22X_2+0.39X_4+0.26X_5+0.086X_7+0.096X_8-0.40X_{10}-0.33X_{11} \quad 1$$

$$Y_2=153.62-12.13X_1-12.00X_2+27.75X_4+22.22X_5+0.29X_7+10.07X_8-19.79X_{10}-11.78X_{11} \quad 2$$

$$Y_3=25.28+3.34X_1+1.00X_2+0.43X_4-0.33X_5+0.16X_7-0.084X_8-0.20X_{10}+1.80X_{11} \quad 3$$

According to statistical analysis of the data, the R^2 of Y_1, Y_2 and Y_3 were 0.9956, 0.9775 and 0.9968, respectively. As shown in Table 4, the p values were 0.0019, 0.0212 and 0.0012 ($p<0.05$) for model of Y_1, Y_2 and Y_3, respectively. Those results indicated that each model exhibited a high degree of fit and reliability. The p value (Table 4) and Fig 1a found that the effect of X_4 and X_{10} on TP of FEAP were very significant ($p<0.001$), while that of X_2, X_5 and X_{11} were significant ($p<0.01$) among eight variables. It was suggested that the TP of FEAP was importantly influenced by X_4 (positive effect) and X_{10} (negative effect). As shown in *p* value (Table 4) and Fig 1b, with EV as the response, X_4 showed significant influence ($p<0.001$) on EV, while X_5 and X_{10} was significant effected ($p<0.05$). Based on the analysis of CP results, X_1 and X_{11} exhibited an extremely significant influence on CP, indicating that CP of FEAP was highest influenced by inorganic nitrogen source addition, followed by W/M. The total nitrogen content of FEAP was increased due to the increasing of the addition amount of urea. Thus, the content of total nitrogen in FEAP (dry base) was increased. Therefore, four variables (bran, glucose, PQ, W/M) were selected for the following experiments.

Screening of the center-points for BBD by using path of steepest ascent method

Eq. 1 and 2 showed that the positive coefficient of X_4 and X_5 and negative coefficient of X_{10} and X_{11}, suggesting that the increasing of bran and glucose and decreasing of PQ and W/M would show positive effects on TP and EV of EFAP. Therefore, the addition amount of bran and glucose in -1 level of PBD for the initial value, with respectively 2 and 1 step length increased gradually, PQ and W/M in 1 level of PBD for the initial value, with respectively 5 and 0.5 step gradually decreased, while the other factors were kept at the -1 level of PBD. With PQ and W/M were 5 g and 1, the fermentation substrate of a 200 mL Erlenmeyer flask were not enough to meet the growth of mixed strains. Thus, the minimum PQ and W/M were 10 g and 1.5 in the path of steepest ascent experiment, respectively. The experimental design and corresponding results were given in Fig 2. $x+\Delta x$ represented 10% bran, 5% glucose, 25 g PQ and 3 W/M, $x+2\Delta x$ represented 12% bran, 6% glucose, 20 g PQ and 2.5 W/M, $x+3\Delta x$ represented 14% bran, 7% glucose, 15 g PQ and 2 W/M, and $x+4\Delta x$ represented 16% bran, 8% glucose, 10 g PQ and 1.5 W/M. LAB viable count of $x+\Delta x$ was higher than that of others, indicating that LAB could grow well under high moisture content substrate. High moisture content of substrate would accelerate the growth and propagation of LAB (16). However, the EV of *A. niger* increased with the decreased of W/M, indicating that *A. niger* was more suitable for growth in low moisture content of the substrate. The TP content of $x+4\Delta x$ was highest among all others, suggesting that the four mixed strains could collaborative symbiosis to promote the accumulation of their own MP. The CP content showed a decreasing trend as W/M gradually decreased. The main reason that the moisture content in the substrate decreased, and the amount of inorganic nitrogen added decreased. Therefore, 16% bran, 8% glucose, 10 g PQ and 1.5 W/M were selected as the intermediate levels of BBD.

Optimization of significant factors by Box-Behnken design

The addition amount of bran (X_4), glucose (X_5), PQ (X_{10}) and W/M (X_{11}) were considered for further optimization using BBD. The center-points chosen for the factors were set as coded value zero based on the path of steepest ascent experiments. The matrix for BBD and the experimental results were shown in Table 5. By applying multiple regression analysis on the experimental data, the responses (TP, EV, CP) could be expressed in terms of following regression equations (Eq. 4, 5 & 6):

$$Y_1=10.15+0.21X_4+0.19X_5-0.066X_{10}-0.20X_{11}-0.016X_4X_5+0.21X_4X_{10}+0.15X_4X_{11}+0.19X_5X_{10}-0.077X_5X_{11}-0.029X_{10}X_{11}-0.59X_4^2-0.48X_5^2-0.34X_{10}^2-0.46X_{11}^2 \quad 4$$

$$Y_2=199.55-4.78X_4-8.73X_5-5.82X_{10}+52.50X_{11}+18.91X_4X_5+22.95X_4X_{10}+14.59X_4X_{11}-18.16X_5X_{10}+4.90X_5X_{11}+27.04X_{10}X_{11}-35.63X_4^2-24.27X_5^2-25.23X_{10}^2-71.15X_{11}^2 \quad 5$$

$$Y_3=18.54+0.041X_4-0.072X_5+0.096X_{10}+1.35X_{11}-0.22X_4X_5+0.39X_4X_{10}-0.24X_4X_{11}-0.44X_5X_{10}-0.16X_5X_{11}-0.58X_{10}X_{11}-0.31X_4^2-0.11X_5^2-0.28X_{10}^2+0.48X_{11}^2 \quad 6$$

As shown in Table 6, *p* values (<0.05) of all models indicated that the model exhibited significant influence on the response value, and the lack of fit (>0.05) indicated that each model had a high degree of fitting. Linear terms X_4, X_5, X_{11}, and quadratic terms X_4^2, X_5^2, X_{10}^2, X_{11}^2 were significant effected

($p<0.05$) for TP. X_{11} and X_{11}^2 exhibited significant influence ($p<0.05$) on EV. Linear term X_{11}, interactive term $X_{10}X_{11}$, and quadratic term X_{11}^2 were significant influenced for CP.

Each three-dimensional surface plot described the effect of two parameters on the responses (TP %, EV ×10^7 CFU/g, CP %), keeping other factors at their zero levels. Those plots were shown in Fig 3A-I. As well as main effect plots of the four factors on the responses were shown in Fig 4A-C. Bran was the main significant effect on the TP content of FEAP ($p<0.05$) (Fig 3A and Fig 4A), while interaction of the four factors on TP were not observed (Table 6). According to Table 6 and Fig 4B-C, bran was not significant effect on EV and CP of FEAP ($p>0.05$). The values of TP, EV and CP were increased and then decreased with increasing amounts of bran. A medium addition of bran showed the highest TP, EV and CP. The ratio of carbon to nitrogen (C/N) in a high level addition of bran might not be a suitable level for the growth of mixed strains.

Glucose was significant effect on the TP of FEAP ($p<0.05$) (Fig 3D, 3G, and Fig 4A), while as to Table 6 and Fig 4B-C, glucose was not significant effect on EV and CP of FEAP ($p>0.05$). The values of TP and EV were increased and then decreased with increasing amounts of bran, while the CP was little influenced. Glucose was mainly used as a carbon source, and changes of glucose would not result in significant changes of nitrogen in the substrate. TP of a high level of glucose was lower than that of a low level, indicating that the four strains was not symbiotically growth well under an unbalanced C/N.

According to Table 6 ($p>0.05$), Fig 3 and Fig 4, PQ was not significant effect on TP, EV and CP. W/M exhibited significant influence on TP, EV and CP of FEAP ($p<0.05$) (Fig 3A, 3H and 3I), especially on CP. The values of TP, EV were increased and then decreased, while CP content increased with increasing of W/M. Those results indicated that the higher fermentation substrate moisture content, the higher CP content. TP of a high level of W/M was lower than that of a low level (Fig 4A), indicating that the accumulation of MP was inhibited by high moisture content. Excessive amount of water added caused insufficient oxygen in fermentation system, influencing the symplastic growth of mixed strains. EV of a high level of W/M was higher than that of a low level (Fig 4A), indicating that LAB could grow well under high moisture content to improve the EV of FEAP.

The point prediction feature of BBD had been used to determine optimum levels of each variable for maximum TP (%), EV (CFU/g), and those were as follows: bran 16.22%, glucose 8.09%, PQ 9.88 g and W/M 1.56. Under those optimized conditions, predicted TP, EV and CP were 10.15%, 2.03×10^9 CFU/g and 18.70%, respectively.

Verification of experimental design

Verification experiment was carried out according to the medium compositions optimized by the software. The measured responses (TP, EV and CP) values were 10.42%, 1.94×10^9 CFU/g and CP 18.73%, which were close to the predicted values. The relative errors of each response value and the predicted value were 2.66%, 4.43% and 0.02% respectively, all within the allowable error range of 5%. It could be seen that response surface models were feasible to optimize the medium for bioconversion EAP to produce microbial protein feed.

Fluorescence microscopy

As shown in Fig 5, A-C indicated sample images, and D-E indicated the images were observed under fluorescence microscope with objective ×10. The microstructure of AP was changed by extrusion and fermentation processing. The stained cellulose of AP was relatively integral and tightly arranged with other structure. The AP exhibited smooth surfaces and no broken edges. The stained cellulose of EAP was relatively less compared with that of AP. EAP showed loose and irregular structure. The bulk of the other tissues were broken by the shear force of the extruder. Several researches were investigated to change the structure of fibrous by-products including apple pomace and orange pomace using extrusion technology (12, 17). Most of the cellulose in AP was hydrolyzed by mixed strains and the particle size of FEAP decreased in comparison of AP and EAP.

Discussion

After extrusion pretreatment, more cellulose of EAP was exposed and degraded by cellulase produced by *A.niger* (31), thus promoting the bioconversion of EAP into MP feed. Madrera et al (19) found that the EV of fermented AP with autochthonous yeasts was ~10^8 CFU/mL on average and its highest CP content was 5.1%. In this study, under optimized medium conditions, the CP content in FEAP was 18.73% (dry base), its EV was 1.94×10^9 CFU/g (wet base), and its TP content was 10.42% (dry base). The results showed that the extrusion pretreatment exhibited a positive effect on the bioconversion of AP. The CP content of this study was obviously higher than that of other report, which mainly due to the addition of nitrogen source. It was worth mentioning that the CP content of unfermented AP in this study (5.93%) was higher than that of AP in Madrera (19) research (3.5%). Indeed, adjustment of the CP content with inorganic nitrogen increased the MP synthesis (33)..

The mixed strains used in this study included *A.niger*, *C.utilis*, *G.caudidum* and LAB. *A.niger* had the ability to produce cellulase, which converted the cellulose of EAP into fermentable sugars, making it more conducive to the growth and reproduction of other strains (9). *C.utilis* and *G.caudidum* could metabolize a variety of carbon sources and had been frequently used for microbial protein production using various agricultural and industrial by-products (36). However, several studies on MP feed from by-products were concerned only with increasing the crude protein content, while neglecting the flavor and palatability. LAB produced organic acids and bacteriocins, inhibiting gram-positive bacteria and improving the intestinal flora of the animal (4, 13). In this study, FEAP showed the higher feeding value accompanying a pleasant flavor, which obtained by Co-culture of *A.niger*, *C.utilis*, *G.caudidum* and LAB.

Combination of PBD and BBD was used to optimize the fermentation medium compositions, in order to make the medium more suitable for the growth of mixed strains. The test results showed that bran exhibited significant influence on the TP, EV and CP of FEAP. The main reason was that bran, as a carbon source and a solid support, loosen the solid substrate and overcame the agglomeration of the substrate, causing better oxygen supply for the growth of mixed strains (32). Several reports had found that monosaccharide glucose was the best carbon source for yeast growth (21, 35). Excessive amount of PQ caused insufficient contact between fermentation substrate and strains, while too small amount of PQ and insufficient nutrients to meet the growth needs of mixed strains. W/M showed significant influence on TP, EV and CP. The changes of W/M influenced the substrate moisture content, which affected the growth of mixed strains and the oxygen supply, gas exchange in the fermentation system (34). High moisture content led to low oxygen content in the fermentation substrate, LAB could carry out anaerobic fermentation. However, the growth of *A. niger* was inhibited by low oxygen content and the large growth of LAB. The increase of W/M led to the increase of total mass of fermentation substrate, thereby increasing the addition amount of inorganic nitrogen. Therefore, the CP was decreased with the decreasing of W/M.

Conclusion

To identify significant factors by screening important variables for MP production by mixed strains (*A. niger*, *C. utilis*, *G. candidum* and LAB). Eight variables had been tested using PBD, which resulted in four significant factors (bran, glucose, PQ and W/M). BBD had been applied for optimization of those factors. The quadratic model had been developed which accurately predicts the levels of variables for maximum TP and EV as: bran 16.22%, glucose 8.09%, PQ 9.88 g and W/M 1.56. The model had been verified by further experimentation, and the measured value (TP 10.42%, EV 1.94×10^9 CFU/g and CP 18.73%) had been found to be close to the predicted value at the optimized conditions. The optimal medium compositions could be suitably used for the production MP feed converting EAP with mixed strains.

Declarations

Funding

This work was supported by Shandong Province Key Research and Development Program Project [grant numbers 2019GNC106076].

Author's Contributions

Zhe Yang: Participated in the whole experiment process and drafted manuscript.

Min Zhang and Lijun Jiang: Participated in part of the experimental design and manuscript preparation.

Wenjing Suo, Yuxin Deng and Haijing Zhang: Participated in part of the experimental design and results analysis.

Peng Guo: Contributed to the guidance of experimental design and ameliorated the manuscript.

Hongjun Li: Contributed to the guidance of experimental design and ameliorated the manuscript and provided financial support.

Ethics approval

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

Figure Captions

Fig.1 Effects of independent variables on TP, EV and CP via PBD

Fig.2 The results of path of steepest ascent

Fig.3 Response surface plots of the effect of variable interactions on TP, EV and CP

Fig.4 Main effect plots for TP, EV and CP of FEAP

Fig.5 Comparison of AP, EAP and FEAP (A-C indicated sample images, D-E indicated the images were observed under fluorescence microscope with objective ×10)

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Figures

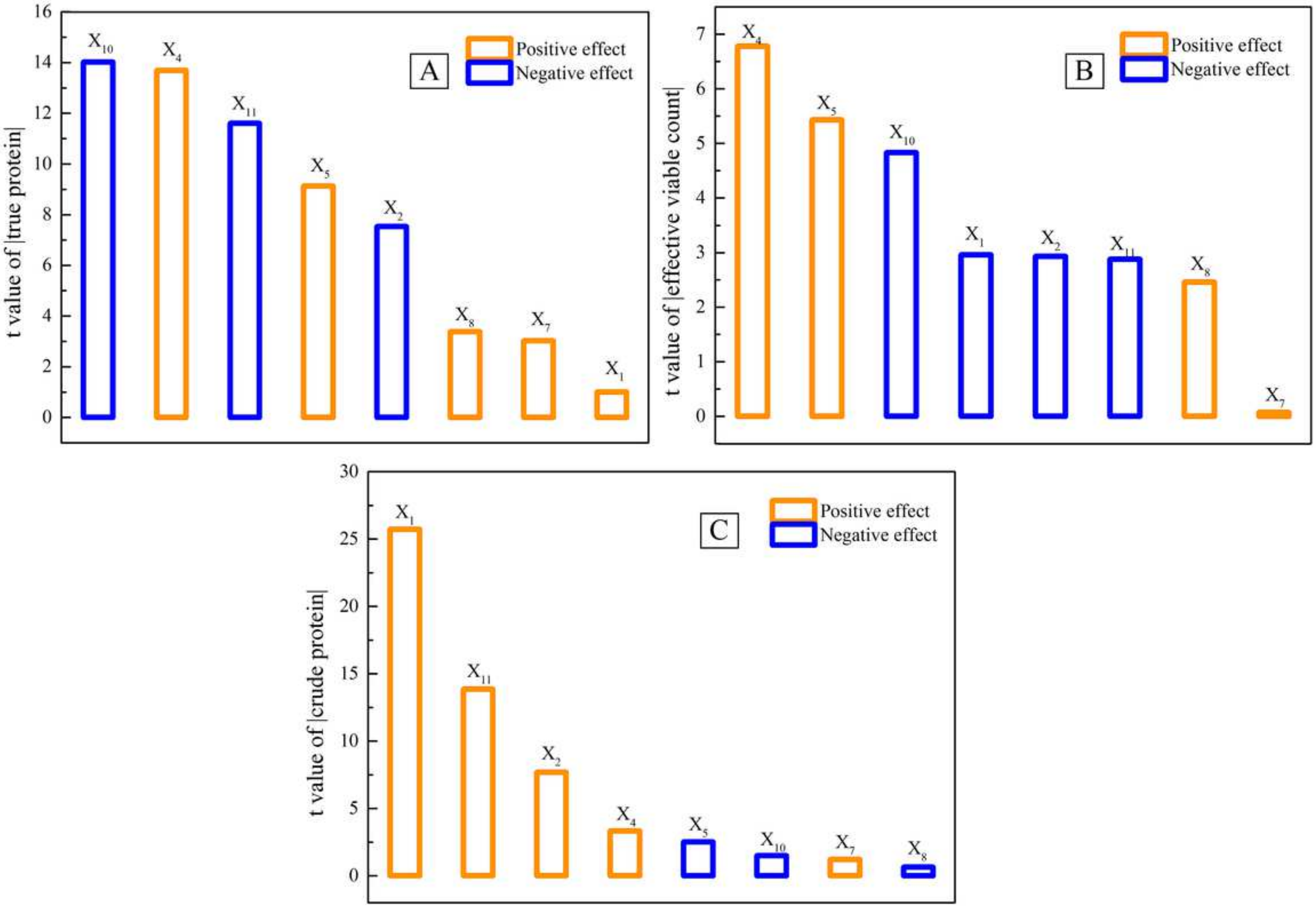

Figure 1

Effects of independent variables on TP, EV and CP via PBD

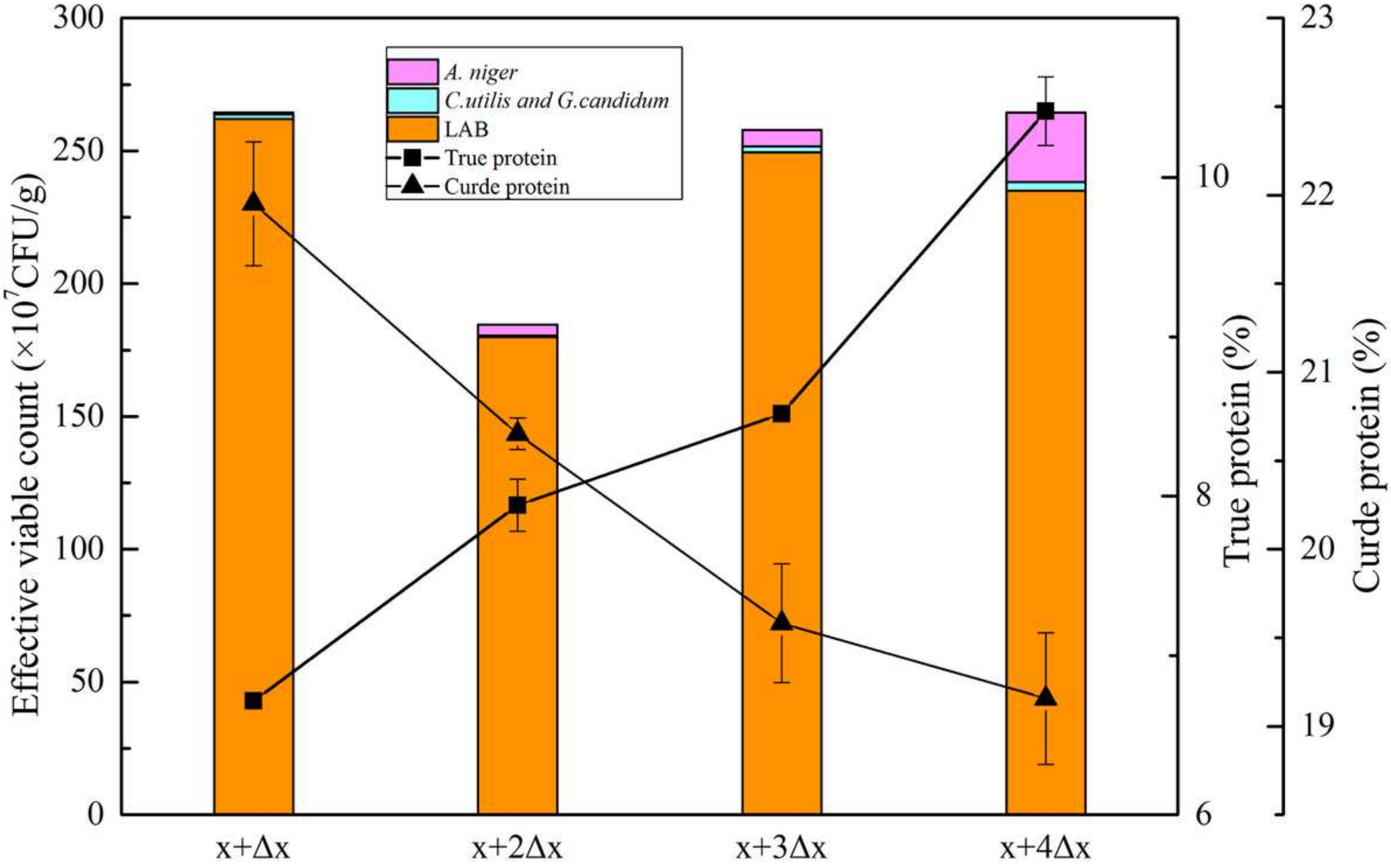

Figure 2

The results of path of steepest ascent

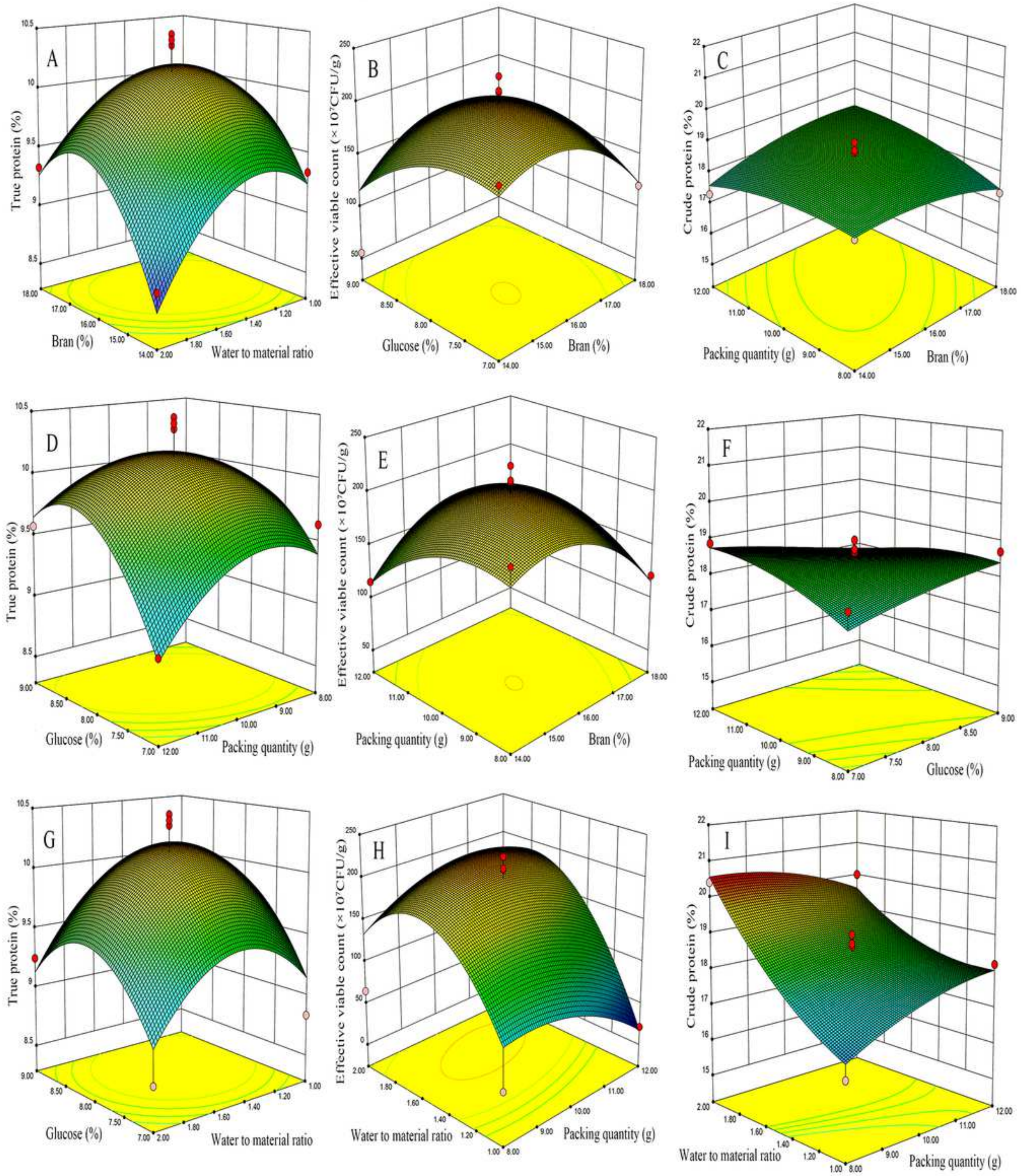

Figure 3

Response surface plots of the effect of variable interactions on TP, EV and CP

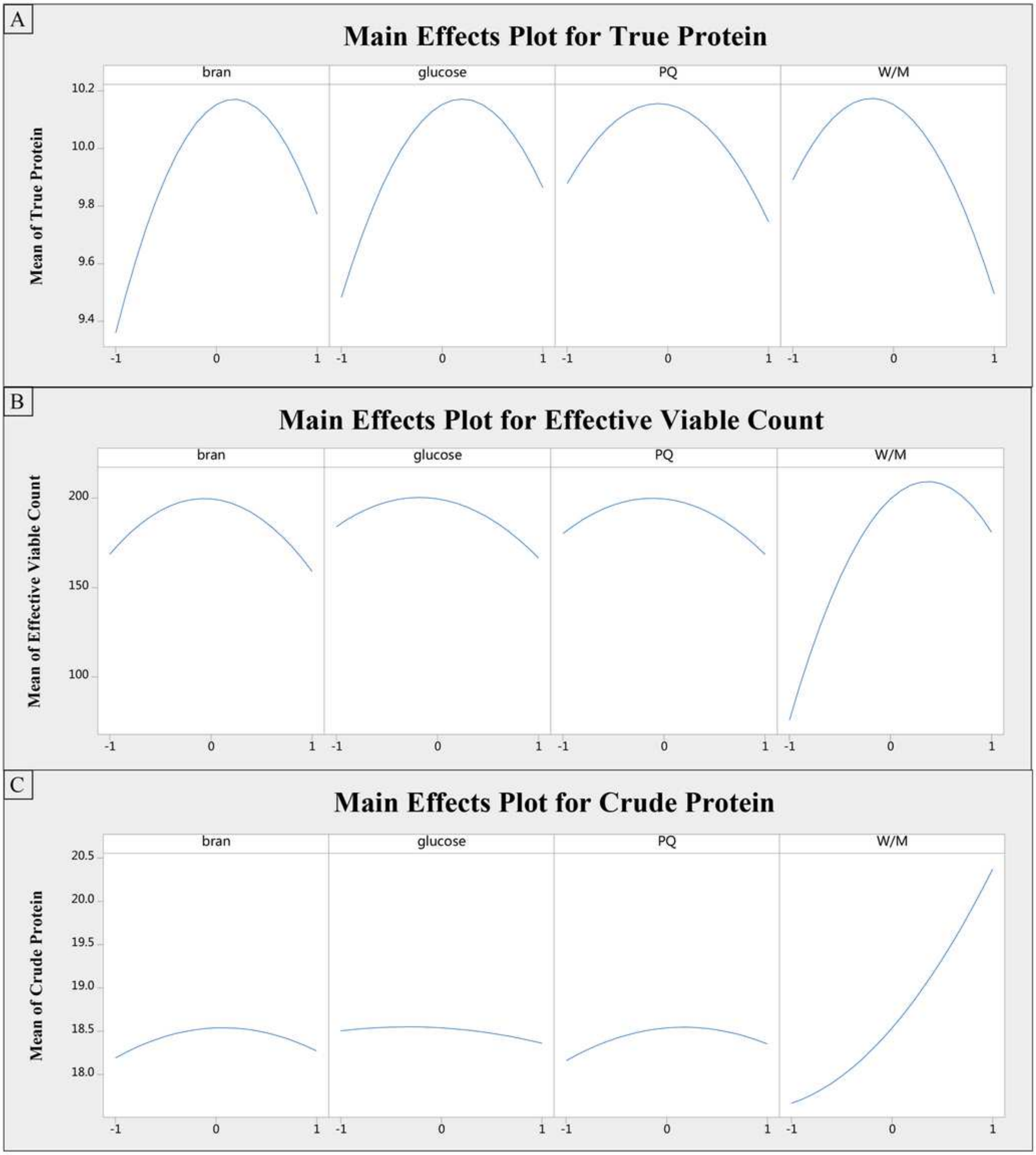

Figure 4

Main effect plots for TP, EV and CP of FEAP

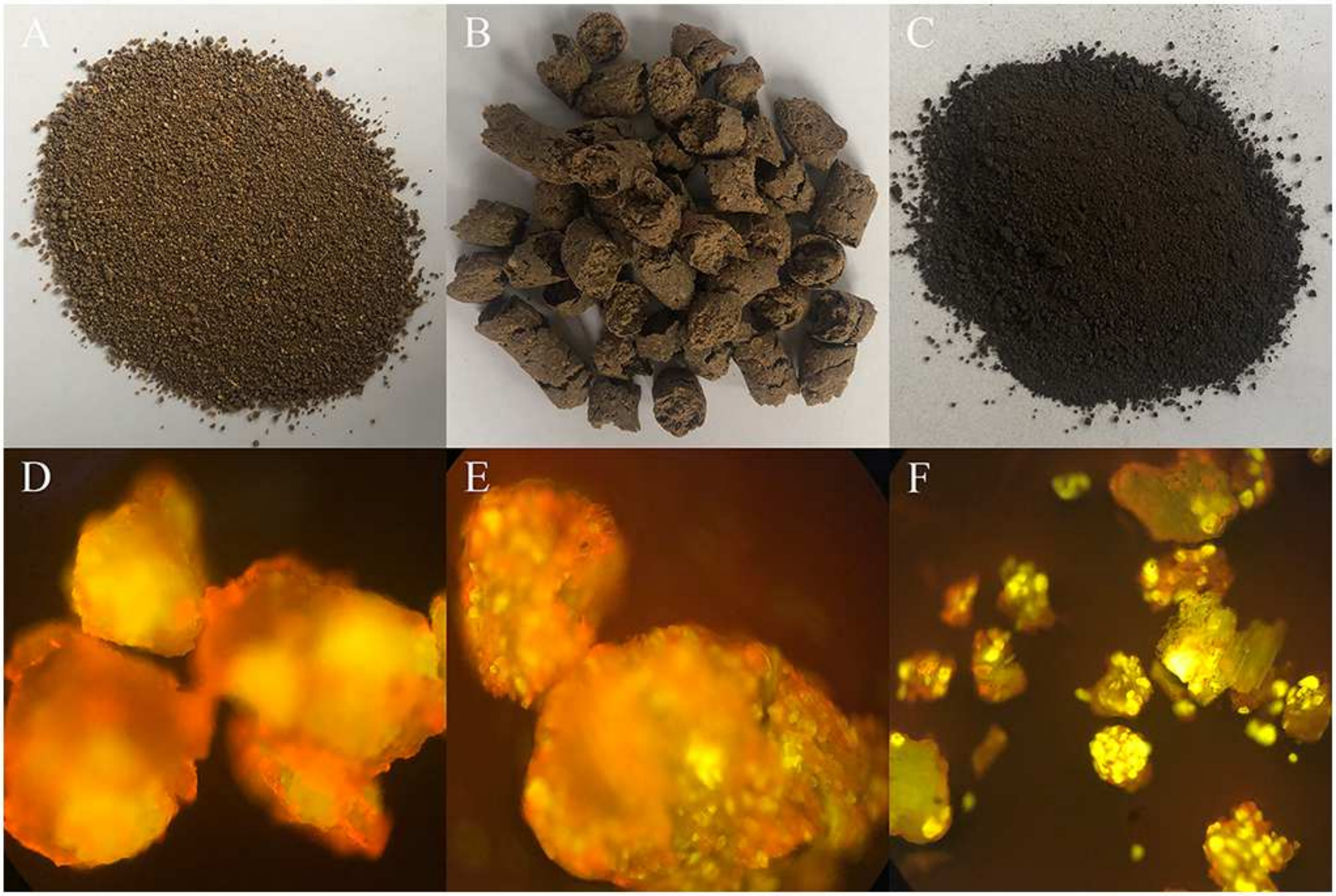

Figure 5

Comparison of AP, EAP and FEAP (A-C indicated sample images, D-E indicated the images were observed under fluorescence microscope with objective ×10)

Supplementary Files

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