Solid-state fermentation of corn by-products mixture feed with *Saccharomyces cerevisiae* , *Lactobacillus plantarum* and protease for improving protein quality and nutrient utilization

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Abstract

Background: Corn germ meal (CGM) and corn gluten feed (CGF) are two main corn by-products (CBs) obtained from corn starch extraction. Due to their high fiber content, low protein content and severe imbalance of amino acid, CBs are unable to be fully utilized by animals. In this study, the effect of microorganism, proteases, temperature, solid-liquid ratio and time on nutritional properties of CBs mixture feed (CMF) was investigated with the single factor method and the response surface method to improve the nutritional quality and utilization of CBs.

Results: Fermentation with Saccharomyces cerevisiae, Lactobacillus plantarum and neutral protease notably improved the nutritional properties of CMF under the fermentation conditions of 37 °C, solid-liquid ratio (1.2:1 g/mL) and 72 h. The crude protein (CP) and trichloroacetic acid soluble protein (TCA-SP) in fermented CMF (FCMF) were increased（ $P < 0.05$）by 14.28% and 25.53%, respectively. The in vitro digestibility of CP and total amino acids of FCMF were significantly improved to 78.53% and 74.94%, respectively. In addition, fermentation degraded fiber and provided more organic acids in the CMF.

Conclusions: Our results suggest that solid-state fermentation with Saccharomyces cerevisiae , Lactobacillus plantarum and protease can efficiently improve protein quality and nutrient utilization of CMF.

Background

The competition for food between human and livestock has become a topic with great concern in the past few years. The shortage of feed resources led to a sharp rise in the prices of conventional feed ingredients, such as corn, soybean meal, fish meal, etc. In order to reduce feed costs, less-expensive, alternative agricultural industrial by-products are increasingly included in livestock diets [1]. Corn is one of the most important cash crops in the world. Global corn production exceeded 1 billion tons per year and the corn processing industry produced large quantities of by-products [2].

Corn germ meal (CGM) and corn gluten feed (CGF) are two main by-products obtained from corn starch extraction by wet milling (Fig. 1). CGM, the remaining portion of corn germ after oil removal, contains 30% protein, 18% starch, 12% cellulose, 2% ash, and 0.7% fat [3]. CGF is produced by combining concentrated steepwater with the fiber during wet milling separation process, which typically contains 60% fiber and 20% protein. The low solubility and imbalanced amino acid (AA) composition of protein limit the application of CBs in feed industry [4].Solid-state fermentation in feedstock processing is an efficient biotechnological approach to improve nutritional value and increase nutrient bioavailability [5, 6]. *Bacillus subtilis*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* were widely used fermentation strains, which inoculated in feedstuff can produce many beneficial factors such as functional peptides, enzymes and organic acids [7]. Our previous research shows that solid fermented soybean meal-corn mixture feed and distilled dried grain with soluble can effectively improve their nutritional value, increase the content of small peptides and lactic acid, and thereby improve growth performance of pig[8–10]. It is also

reported that enzymatic modification of zein can improve its water solubility and utilization [11]. However, few comparisons had been conducted to evaluate effect of different strain and enzyme on solid-state fermented CBs.

In this study, a solid-state fermented feed system contained CBs, corn, soybean meal and wheat bran was performed to gain a sufficient usage of CBs. To degrade fibers and produce digestible protein, *Bacillus subtilis*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* were adopted in fermentation and reinforced by adding protease. Fermentation time, liquid ratio and temperature would be traced to optimal fermentation conditions and form a fermentation strategy.

Methods

Microorganisms and material

Bacillus subtilis ZJU12-1 (CGMCC No:12825) and *Lactobacillus plantarum* CWLP (CGMCC No:1.510) were obtained from Chinese traditional pickled vegetables. *Saccharomyces cerevisiae* WFSC (NCBI accession number: MN038413) was obtained from Chinese local distiller's grains. *Bacillus subtilis* ZJU12-1, *Lactobacillus plantarum* CWLP and *Saccharomyces cerevisiae* WFSC were maintained on Luria broth (LB) plates, de Man, Rogosa and Sharp (MRS) plates and Yeast Extract Peptone Dextrose (YPD) plates preserved at 4 °C. Neutral protease from *Bacillus spp*. (P3111; Sigma-Aldrich Corp.). CMF contains 20% CGM, 30% CGF, 30% corn, 15% soybean meal and 5% wheat bran, which were obtained from the Cofine Bio - tech Co., Ltd. (Jiaxing, China).

Selection of fermentation combinations

The fermentation combinations were set as follows to obtained the optimal fermentation combination: *B. subtilis* ZJU12-1 + *S. cerevisiae* WFSC, *S. cerevisiae* WFSC + *L. plantarum* CWLP, *B. subtilis* ZJU12-1 + *L.plantarum* CWLP, *S. cerevisiae* WFSC + neutral protease, *L. plantarum* CWLP + neutral protease, *L. plantarum* CWLP + *S. cerevisiae* WFSC + neutral protease. Before fermentation, *B. subtilis* ZJU12-1 was cultured in Luria broth (LB) liquid medium at 37 °C for 12 h. *L. plantarum* CWLP was cultured in de Man, Rogosa and Sharp (MRS) liquid medium at 37 °C for 18 h. *S. cerevisiae* WFSC was cultured in Yeast Extract Peptone Dextrose (YPD) liquid medium at 37 °C for 12 h. The 100 g CMF was mixed and placed in a 500 mL Erlenmeyer flask, and sterile water was added to achieve a solid-liquid ratio (1.5:1 g/mL). The wet mixed CMF was inoculated with probiotic (7.0 log cfu/g) or protease (50 U/g) according to the fermentation combinations listed above. Then the flask was covered with a membrane (aerobic condition) and fermented at 37 °C. After 72 h of fermentation, wet samples were collected for testing microorganisms and microbial metabolites. The rest of the samples were treated at 105 °C for 30 min to prevent continuous fermentation. Then, samples were dried at 65 °C for 24 h, cooled and ground into a fine powder for further analyses.

Optimization of fermentation conditions

Based on the restriction of the fermentation conditions of solid state fermentation in actual scale production in China, the solid-liquid ratio (1.8:1, 1.5:1 and 1.2:1 g/mL), fermentation temperature (27 °C, 32 °C, 37 °C), fermentation time (24 h, 48 h, 72 h) were investigated with response surface analysis to optimize the parameters of the optimal fermentation, and the Design Expert software (Version 8.0.6, Stat-Ease. Inc, Minneapolis, MN. USA) was used for the regression and graphical analysis of the experimental data obtained.

$M = a0 + a1 \times A + a2 \times B + a3 \times C + a4 \times A^2 + a5 \times B^2 + a6 \times C^2 + a7 \times A \times B + a8 \times A \times C + a9 \times B \times C$. M is the predicted response; a0 is the intercept term; a1, a2, a3 are the linear coefficients; a4, a5, a6 are the squared coefficients; a7, a8, a9 are the interaction coefficients.

Chemical analyses

The dried samples of CMF were ground, sieved through a 1 mm sieve, and then the dry matter (DM), crude protein (CP), amino acid (AA), ether extract (EE), crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed as described by AOAC (2005). Determination of trichloroacetic acid soluble protein (TCA-SP) in samples was using the method proposed by Ovissipour et al [12].

Microorganisms and microbial metabolites

The pH and microbial counts were analyzed by the method of C. Wang et al. [8] with minor modifications. In brief, 5 g wet samples were dissolved in 45 mL sterile water and placed on a shaker at 150 rpm for 20 min. The pH of the supernatant was measured with a pH meter (Mettler Toledo, Switzerland). The samples were diluted 10-fold with sterile water for microbial counts. The viable count of *B. subtilis* was counted after culturing on LB agar for 24 h at 37 °C. The viable count of *L. plantarum* was counted after culturing on MRS agar for 48 h at 37 °C. The viable count *S. cerevisiae* was counted after culturing on YPD agar for 48 h at 37 °C.

The concentration of organic acids (acetic acid, propionic acid, butyric acid and lactic acid) in each sample was separated and quantified using a gas chromatograph (GC; GC-14B, Shimadzu, Japan; capillary column 30 m × 0.32 mm × 0.25 μm film thickness/VARIAN CP-3800, Varian, Palo Alto, CA, USA) as described by Khajeh et al. [13]. In brief, the samples (1 g) were thawed and suspended in 2-ml of distilled water in a screw-capped tube. After being vortexed, each sample was centrifuged (12000 × g) at 4 °C for 10 min. The supernatant (1-ml) was transferred into a 2 ml centrifuge tube and mixed with 0.2 ml metaphosphoric acid and kept at 4 °C for 30 min. The mixtures were then centrifuged (12000 × g) again at 4 °C for 10 min. Aliquots of the supernatant (1 μl) were analyzed by GC.

In vitro **digestibility**

In vitro two-stage enzymatic hydrolysis process was performed by the method of Sakamoto et al. [14] with minor modifications. In short, CMF or FCMF (2 g) was added to a 150 mL Erlenmeyer flask, containing 50 ml, 10000 U/mL pepsin (activity: 3000 U/mg, Sigma) solution (0.05 mol/L KCl-HCl buffer, pH 2.0) and incubated on a shaker at 37 ℃, 100 rpm for 5 h. The pH of the mixture was then adjusted to

6.8 with 1 mol/L NaOH and 1 mol/L HCl, and 150 mg trypsin (activity: 250 U/mg, Sigma) was added to the mixture and incubated on a shaker at 37 °C, 100 rpm for 5 h. After digestion, 5 mL 20% sulfosalicylic acid was added to the mixture and settled for 30 min. The digesta slurry samples were centrifuged at 3,000 × g for 15 min. The precipitate, washed with double steaming water for several times and collected, dried at 105 °C, was used to analyze the content of CP and AA. *In vitro* CP(AA) digestibility (%) = (original CP (AA) amount – residual CP (AA) amount) / original CP (AA) amount × 100%.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins in CMF and FCMF were extracted by using the procedure described by Faurobert [15]. The gel running conditions were chosen according to the report of Meinlschmidt et al. [16]. In brief, the gal was stained with Coomassie Brilliant Blue R-250 (Bio-Rad, USA) for 2 h and de-stained with 7% acetic acid.

Microscopic analyses

Proteins were labeled with fluorescein isothiocyanate (FITC), lignocellulose was labeled with calcofuor white (CW), and starch was labeled with Concanavalin A (Con A) [17]. CLSM was performed by the method of Zhang et al. with minor modifications [10]. In brief, 10 g/L FITC solution, 250 mg/L Con A solution and 300 mg/L CW solution were added to the wet samples for 1 h, 30 min and 30 min, respectively. After each staining, the samples were washed three times with deionized water. Stained samples were placed on glass slides and observed under a confocal laser scanning microscope (LSM 710; Carl Zeiss MicroImaging GmbH, Jena, Germany). The microstructure of the sample was observed with ZEN 2010 software (Carl Zeiss MicroImaging GmbH). Protein, fiber and starch exhibit green fluorescence, blue fluorescence and red fluorescence, respectively. Fluorescence intensity indicates the content of each nutrient.

Statistical analysis

All assay data were analyzed using SPSS 20.0 software (SAS Inc., Chicago, IL). One-way ANOVA and Duncan test were used to determine the difference between the means values, and data are expressed as mean value ± standard deviation. The differences between the treatments' means were considered significant at $P < 0.05$.

Results

Selection of the strain and enzyme

Based on the results of the single factor experiment (Table 1), CMF fermented with *S. cerevisiae* WFSC, *L. plantarum* CWLP and neutral protease (CMFSLN) has the highest content of CP, TCA-SP and EE compared to the other fermentation combinations, which were 9.52%, 22.14% and 38.12% higher than that of CMF, respectively. Furthermore, CF of CMFSLN is significantly lower than that of CMF ($p < 0.05$).

These results indicate that the process of fermentation with *S. cerevisiae* WFSC, *L. plantarum* CWLP and neutral protease greatly improved the nutritional composition of CMF. In addition, CMFSLN has a larger viable count of *S. cerevisiae* WFSC and *L. plantarum* CWLP. The results above all suggest that CMFSLN may have the greatest fermentation potential.

Table 1

Nutrient composition and microorganisms of CMF and CMF fermented with different probiotics and neutral protease (as air-dry basis)

Items	CMF	CMFBS	CMFSL	CMFBL	CMFSN	CMFLN	CMFSLN
DM,%	90.33 ± 0.40	91.46 ± 0.48	91.08 ± 0.42	90.84 ± 0.56	90.80 ± 0.36	91.96 ± 0.22	91.04 ± 0.17
EE,%	2.23 ± 0.04^{e}	2.51 ± 0.08^{d}	2.59 ± 0.03 ^{cd}	2.72 ± 0.06^{c}	2.88 ± 0.08^{b}	2.36 ± 0.08^{e}	3.08 ± 0.14^{a}
CP,%	22.27 ± 0.35^{d}	23.60 ± 0.40^{b}	23.42 ± 0.48^{bc}	22.85 ± 0.25^{c}	23.08 ± 0.14^{bc}	23.29 ± 0.28^{bc}	24.39 ± 0.24^{a}
TCA-SP,%	37.45 ± 0.47^{e}	44.22 ± 0.31^{bc}	42.78 ± 0.27^{d}	43.65 ± 0.39^{c}	44.00 ± 0.29^{c}	44.72 ± 0.24^{b}	45.74 ± 0.13^{a}
CF,%	6.11 ± 0.32^{a}	5.33 ± 0.39^{bc}	5.51 ± 0.42^{b}	5.18 ± 0.14^{bc}	5.37 ± 0.32b^{c}	5.12 ± 0.10^{bc}	4.91 ± 0.16^{c}
pH	6.40 ± 0.03^{a}	6.22 ± 0.10^{a}	4.67 ± 0.09^{b}	4.69 ± 0.17^{b}	6.21 ± 0.12^{a}	4.55 ± 0.07^{b}	4.52 ± 0.18^{b}
Microorganism, ×10^{7} CFU/g							
B. subtilis ZJU12-1	-	82.00 ± 5.13	-	5.33 ± 8.19	-	-	-
S. cerevisiae WFSC	-	1.80 ± 0.61	2.10 ± 0.44	-	1.71 ± 0.61	-	2.73 ± 0.65
L. plantarum CWLP	-	-	16.67 ± 4.51	14.67 ± 4.51	-	16.00 ± 0.44	23.00 ± 6.25
CMFBS, CMF fermented with *B. subtilis* ZJU12-1 and *S. cerevisiae* WFSC							
CMFSL, CMF fermented with *S. cerevisiae* WFSC and *L. plantarum* CWLP							
CMFBL, CMF fermented with *B. subtilis* ZJU12-1 and *L. plantarum* CWLP							
CMFSN, CMF fermented with *S. cerevisiae* WFSC and neutral protease							
CMFLN, CMF fermented with *L. plantarum* CWLP and neutral protease							
CMFSLN, CMF fermented with *S. cerevisiae* WFSC, *L. plantarum* CWLP and neutral protease							
Values were mean ± SD, n = 3. Means followed with different superscript letters (a, b) within each line are significantly different ($p < 0.05$)							

Optimal fermentation conditions of CMFSLN

Box-Behnken Design (BBD) was employed to optimize three variables: fermentation temperature (A), fermentation time (B) and solid-liquid ratio (C) of CMFSLN. Based on the response surface results (Fig. 2) and multiple regression analysis of experimental data, the following second order polynomial equation between the viable count of *S. cerevisiae* WFSC, *L. plantarum* CWLP and three variables during fermentation were found: the viable count of *S. cerevisiae* WFSC = $6.56 + 0.27 \times A + 0.38 \times B + 2.20 \times C + 0.64 \times A \times B + 0.025 \times A \times C - 0.015 \times B \times C - 3.08 \times A^2 - 2.72 \times B^2 - 1.31 \times C^2$, the viable count of *L. plantarum* CWLP = $28.80 - 1.3 \times A + 8.89 \times B + 27.36 \times C + 3.38 \times A \times B + 13.83 \times A \times C + 7.10 \times B \times C - 6.55 \times A^2 - 0.13 \times B^2 + 15.42 \times C^2$. Similarly, second order polynomial equation between the content of CP, TCA-SP and three variables during fermentation were found: the content of CP = $23.41 + 0.47 \times A + 0.34 \times B + 0.65 \times C - 0.046 \times A \times B + 0.52 \times A \times C + 0.66 \times B \times C - 0.60 \times A^2 + 0.20 \times B^2 - 0.22 \times C^2$, the content of TCA-SP = $40.63 - 1.45 \times A - 1.35 \times B - 1.46 \times C - 0.59 \times A \times B - 2.76 \times A \times C - 1.14 \times B \times C + 1.73 \times A^2 + 2.06 \times B^2 - 0.91 \times C^2$.

By solving the regression equations above, the optimal condition of the three variables to obtain the maximum point of the model were calculated to be: fermentation temperature 32 ℃, fermentation time 50 h and solid-liquid ratio 1.2:1 g/mL, with the corresponding viable count of *S. cerevisiae* WFSC 7.50×10^7 CFU/g. Similarly, under the optimal conditions of fermentation temperature 37 ℃, fermentation time 72 h and solid-liquid ratio 1.2:1 g/mL, the viable count of *L. plantarum* CWLP reached the maximum value of 96.75×10^7 CFU/g. The content of CP reached the maximum value of 25.86%, which was the same as the optimal conditions for the maximum viable count of *L. plantarum* CWLP. In addition, under the optimal conditions of fermentation temperature 37 ℃, fermentation time 24 h and solid-liquid ratio 1.8:1 g/mL, the content of TCA-SP reached the maximum value of 46.93%. In order to verify the optimization results, a verification experiment was conducted to show that the viable count of *S. cerevisiae* WFSC, *L. plantarum* CWLP, the content of CP and TCA-SP in their optimum conditions were 7.82×10^7 CFU/g, 90.21×10^7 CFU/g, 25.52% and 46.21% respectively, which indicated that the model was satisfactory and practicable.

Chemical composition of FCMF

The nutrient contents of CMF and FCMF are presented in Table 2. Compared with CMF, the fermented CMF contained more CP, TCA-SP and EE, which were augmented ($P < 0.05$) by approximately14.28%, 25.33% and 42.119%, respectively. The content of CF, ADF and NDF were decreased ($P < 0.05$) by 29.10%, 10.43% and 18.15%, respectively. In this study, the content of amylose and total starch were decreased greatly after fermentation ($P < 0.05$).

Table 2

Nutrient composition of CMF and FCMF (as air-dry basis)

Items	CMF	FCMF	Items	CMF	FCMF
DM,%	90.33 ± 0.02[a]	91.67 ± 0.02[a]	Indispensable AA, %		
EE,%	2.28 ± 0.14[b]	3.24 ± 0.11[a]	Arg	1.74 ± 0.02[a]	1.75 ± 0.01[a]
CP,%	22.33 ± 0.13[b]	25.52 ± 0.18[a]	His	0.59 ± 0.02[b]	0.66 ± 0.02[a]
TCA-SP,%	37.26 ± 1.00[b]	46.70 ± 0.78[a]	Ile	1.38 ± 0.01[b]	1.55 ± 0.06[a]
CF,%	6.70 ± 0.17[a]	4.05 ± 0.09[b]	Leu	0.74 ± 0.01[a]	0.76 ± 0.00 [a]
ADF,%	10.64 ± 0.08[a]	9.53 ± 0.17[b]	Lys	0.74 ± 0.01[a]	0.76 ± 0.00 [a]
NDF,%	28.71 ± 0.13[a]	23.50 ± 0.25[b]	Met	0.16 ± 0.01[a]	0.18 ± 0.01[a]
Total starch,%	14.49 ± 0.11[a]	13.96 ± 0.05[b]	Phe	0.75 ± 0.01[b]	0.83 ± 0.03[a]
Amylopectin,%	10.40 ± 0.14[a]	10.34 ± 0.07[a]	Thr	0.38 ± 0.01[a]	0.41 ± 0.01[a]
Amylose,%	4.09 ± 0.05[a]	3.62 ± 0.06[b]	Val	0.78 ± 0.01[a]	0.85 ± 0.05[a]
			Dispensable AA,%		
			Asp	1.74 ± 0.00[a]	1.71 ± 0.01[a]
			Ser	1.41 ± 0.01[b]	1.54 ± 0.00 [a]
			Glu	4.61 ± 0.07[a]	4.76 ± 0.07[a]
			Gly	0.62 ± 0.01[a]	0.64 ± 0.00[a]
			Ala	1.32 ± 0.01[b]	1.48 ± 0.00 [a]
			Cys	0.21 ± 0.01[a]	0.23 ± 0.00[a]
			Tyr	1.19 ± 0.03[a]	1.26 ± 0.05[a]
			Pro	1.41 ± 0.00[a]	1.37 ± 0.03[a]

Values were mean ± SD, n = 3. Means followed with different superscript letters (a, b) within each line are significantly different ($p < 0.05$)

Items	CMF	FCMF	Items	CMF	FCMF
			Total AA	19.61 ± 0.13^{b}	20.67 ± 0.12^{a}

Values were mean ± SD, n = 3. Means followed with different superscript letters (a, b) within each line are significantly different ($p < 0.05$)

In addition, fermentation with probiotics and neutral protease affected the AA composition in CMF. In the present study, three indispensable AA (His, Ile and Phe), two dispensable AA (Ser and Ala) and total AA significantly increased in FCMF compared to CMF. In addition, except for ASP, most AA showed an increase trend after fermentation.

Microorganisms and microbial metabolites of FCMF

To further evaluate the nutritional properties of FCMF, we determined the microorganisms and microbial metabolites after fermentation, and the results are presented in Table 3. After fermentation, the viable counts of *S. cerevisiae* WFSC and *L. plantarum* CWLP in FCMF reached 6.95×10^7 CFU/g and 90.21×10^7 CFU/g respectively. The pH of CMF decreased from 6.42 to 4.54, which was mainly caused by organic acids produced by *L. plantarum* CWLP. The acetic acid, propionic acid, butyric acid, lactic acid in FCMF were increased by 6.72, 20.83, 21.00, 5.64 times, respectively.

Table 3
Microorganism and microbial metabolites of CMF and FCMF

Items	CMF	FCMF
Organic acids, mg/100 g		
Acetic acid	22.07 ± 0.04^{b}	148.22 ± 0.52^{a}
Propionic acid	0.06 ± 0.01^{b}	1.25 ± 0.03^{a}
Butyric acid	0.18 ± 0.01^{b}	3.78 ± 0.09^{a}
Lactic acid	26.53 ± 0.08^{b}	149.61 ± 0.09^{a}
pH	6.42 ± 0.11^{a}	4.54 ± 0.15^{b}
Microorganism,10^7 CFU/g		
S. cerevisiae WFSC	-	6.95 ± 0.60
L. plantarum WCLP	-	90.21 ± 8.40

Values were mean ± SD, n = 3. Means followed with different superscript letters (a, b) within each line are significantly different ($p < 0.05$)

In vitro **digestibility of FCMF**

The results of digestibility of CMF and FCMF are presented in Table 4. The *in vitro* digestibility of DM and CP in FCMF were notably improved by 18.98% and 16.62%, respectively, compared with CMF. In addition, the *in vitro* digestibility of fourteen AA, including nine essential amino acids (Arg, His, Ile, Leu, Met, Lys, Thr, Phe and Val) and five dispensable AA (Ser, Glu, Ala, Tyr and Cys) were significantly enhanced. Furthermore, after fermentation, the digestibility of average indispensable AA, average dispensable AA and total AA were greatly improved approximately 1.19, 1.17 and 1.13 times, respectively.

Table 4
In vitro CP and AA digestibility (%) of CMF and FCMF

Items	CMF	FCMF
DM,%	48.89 ± 0.37[b]	58.17 ± 1.64[a]
CP,%	67.34 ± 0.70[b]	78.53 ± 0.51[a]
Indispensable AA, %		
Arg	64.50 ± 1.02[b]	73.71 ± 4.75[a]
His	66.11 ± 3.13[b]	79.37 ± 3.18[a]
Ile	52.58 ± 1.30[b]	66.33 ± 4.15[a]
Leu	57.25 ± 1.19[b]	64.92 ± 2.19[a]
Lys	50.67 ± 1.53[b]	60.67 ± 3.06[a]
Met	52.81 ± 1.51[b]	58.66 ± 0.60[a]
Phe	63.61 ± 0.54[b]	76.81 ± 1.92[a]
Thr	50.4 ± 1.48[b]	62.21 ± 1.15[a]
Val	51.86 ± 1.47[b]	65.91 ± 2.59[a]
Dispensable AA,%		
Asp	85.53 ± 0.19[a]	81.76 ± 1.59 [b]
Ser	62.57 ± 1.76[b]	74.65 ± 1.10[a]
Glu	79.75 ± 1.51[b]	91.08 ± 1.94[a]
Gly	61.44 ± 0.99[a]	60.44 ± 1.97[a]
Ala	56.46 ± 1.13[b]	67.61 ± 1.96[a]
Cys	49.86 ± 1.29[b]	59.75 ± 4.25[a]
Tys	55.35 ± 3.34[b]	63.55 ± 2.96[a]
Pro	66.91 ± 1.41[a]	68.83 ± 1.56[a]
Total AA,%	66.20 ± 0.31[b]	74.94 ± 1.27[a]

Values were mean ± SD, n = 3. Means followed with different superscript letters (a, b) within each line are significantly different ($p < 0.05$)

The degradation of macromolecular proteins in FCMF

The SDS-PAGE protein profiles of CMF and FCMF are showed in Fig. 3. The protein profile corresponded to multiple bands in the range of 20–100 kDa in CMF. Fermentation with *S. cerevisiae* WFSC, *L. plantarum* CWLP and neutral protease for 48 h, the protein profile corresponded to multiple bands in the range of 55–100 kDa in FCMF were completely degraded. After 72 hours of fermentation, the protein profile corresponded to multiple bands in the range of 30–50 kDa in FCMF were obviously degraded. In addition, the content of small peptides (< 25 kDa) were significantly increased in FCMF compared with CMF.

Microscopic observation of FCMF

The CLSM images of CGM and CGF before and after fermentation are presented in Fig. 4. The fiber structure on the surface of CGM showed blue fluorescence after CW staining, which obviously faded after fermentation. And the protein of CGM, after stained with FITC, showed brighter green fluorescence after fermentation (Fig. 4a and 4b). In addition, the dense fiber structure on the surface of CGF was destroyed to expose internal protein and starch granules after fermentation (Fig. 4c and 4d).

Discussion

In recent years, there have been many reports on the positive effects of probiotics(such as Saccharomyces cerevisiae and *Lactobacillus plantarum*, etc.) and their metabolites in fermented feed. On the one hand, these probiotics secrete a series of enzymes that effectively degrade anti-nutritional factors to improve the nutrient value of feed materials [18]. On the other hand, probiotics and their functional metabolites, such as organic acids, cell wall polysaccharides, etc., can greatly improve the immune function of animals and inhibit the proliferation of pathogenic microorganisms, thereby maintaining the health of the animals [19–21]. *Bacillus.spp*, *Lactobacillus*. s*pp* and yeast were widely used in feed fermentation. However, the organic acids produced by *Lactobacillus*.s*pp* would limit the activity of *Bacillus.spp* and thus inhibit its secretion of proteases[22]. In the present study, we found that fermentation of *S. cerevisiae* WFSC, *L. plantarum* CWLP and neutral protease had the best effect on improving CP and TCA-SP of corn by-products through single-factor experiments, which indicated that the role of *Bacillus.spp* in degrading proteins into TCA-SP could be replaced by neutral proteases.

Response surface analysis is an effective way to investigate the interaction between different factors during fermentation. Compared to previous report[23], the optimized fermentation conditions of soybean meal by response surface analysis were fermentation temperature (30 °C), fermentation time (72 h), solid-liquid ratio (1:3.5 (g/mL)), the protein hydrolysis of fermented soybean meal could reach to 10.05% by *Neurospora crassa* under the fermentation conditions. In our research, under the fermentation conditions of 32 °C, solid-liquid ratio (1.2:1 g/mL) and 50 h, the proliferation of *S. cerevisiae* WFSC reached its maximum. The rapid growth of *S. cerevisiae* WFSC increased the consumption of oxygen, which provide an anaerobic environment for *L. plantarum* CWLP [24]. The viable count of *L. plantarum* CWLP reached

its maximum value under the fermentation conditions of 37 °C, solid-liquid ratio (1.2:1 g/mL) and 72 h. In addition, neutral protease may have the optimal enzyme activity to degrade macromolecular proteins into TCA-SP at 37 °C. Accumulation of single cell protein produced by microorganisms may lead to the increase of CP [25, 26], which reached its maximum under the fermentation conditions of 37 °C, solid-liquid ratio (1.2:1 g/mL) and 72 h. In summary, we recommend that the fermentation conditions of 37 °C, solid-liquid ratio (1.2:1 g/mL) and 72 h for solid-state fermentation of CMF.

The content of CP, TCA-SP and AA in CMF was significantly increased after the fermentation in our research. In addition to the accumulation of single-cell proteins produced by microorganisms, the loss of dry matter (mainly carbohydrates) in the fermentation substrate may be another reason for the relative increase in the concentration of CP [27]. TCA-SP consists of small peptides and free AA, most of which can be directly absorbed by the gastrointestinal tract [28]. In addition, AA composition patterns changes during fermentation may be related to microbial protein synthesis and decomposition, which may suggests an ideal amino acid pattern for animals [29]. Therefore, the increase in the content of TCA-SP and the change in AA composition can improve the nutritional value of CMF. Furthermore, the lignocellulosic components and amylose is poorly digested in the upper gut of monogastric animals [30]. CF, ADF and amylose are effectively degraded after fermentation in this study, which might due to the cellulase and amylase secreted by *S. cerevisiae* WFSC and *L. plantarum* CWLP [31, 32].

Digestibility is an important indicate to evaluate the nutritional value of protein. The increase of CP and TCA-SP and the optimization of AA composition pattern in FCMF may be the main reasons for improving the in vitro digestibility of CP and AA. Some macromolecular proteins induce allergic reactions in humans and animals [33, 34]. In the present study, CMF, fermented with *S. cerevisiae* WFSC, *L. plantarum* CWLP and neutral protease, contained less macromolecular proteins and more small peptides compared with CMF, the result was consistent with the previous report [35].In addition, the degradation of viscous-resistant starch and cellulose in FCMF leads to the exposure of internal proteins to the environment of pepsin and trypsin [36, 37], which possibly contributes to the in vitro digestibility of CP and AA. Pericarp in CGF is mainly composed of cellulose and hemicellulose, which is partially degraded by SO_2 soaking process in wet milling [38, 39]. The process of fermentation with *S. cerevisiae* WFSC, *L. plantarum* CWLP and neutral protease further degraded these fibers [40]. Furthermore, the low pH value of FCMF is more effective to promote the function of pepsin [41].

Conclusions

Solid-state fermentation with *S. cerevisiae* WFSC, *L. plantarum* CWLP and neutral protease under the conditions of fermentation temperature 37 ℃, fermentation time 72 h and solid-liquid ratio 1.2:1 g/mL effectively improve protein digestibility in CMF though degrading macromolecular protein into TCA-SP, improving amino acids composition patterns and degrading lignocellulose to expose internal nutrients. Therefore, this study provides a strategy for the utilization of CBs as feed materials.

Abbreviations

CGM: Corn germ meal; CGF: corn gluten feed; CBs: Corn by-products; CMF: CBs mixture feed; FCMF: Fermented CBs mixture feed; DM: Dry matter; EE: Ether extract; AA: Amino acid; CP: Crude protein; TCA-SP: Trichloroacetic acid soluble protein; CF: Crude fiber; ADF: Acid detergent fiber; NDF: Neutral detergent fiber; SDS-PAGE: Sodium dodecyl sulfate – polyacrylamide gel electrophoresis.

Declarations

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Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available. Please contact the authors for data requests.

Author's contributions

YZ and WFS conceived and designed the experiment. WFS, CW, ZPJ, LHH and THW carried out the solid-state fermentation, chemical analysis, microscopic analyses, SDS-PAGE and determination of in vitro digestibility. FQW carried out the determination of organic acids. WFS analyzed the data and wrote the manuscript. YZW, ZQL,TZZ verified the validity and checked the results. All authors read and approved the final version of this manuscript.

Competing interests

The authors have declared no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Figures

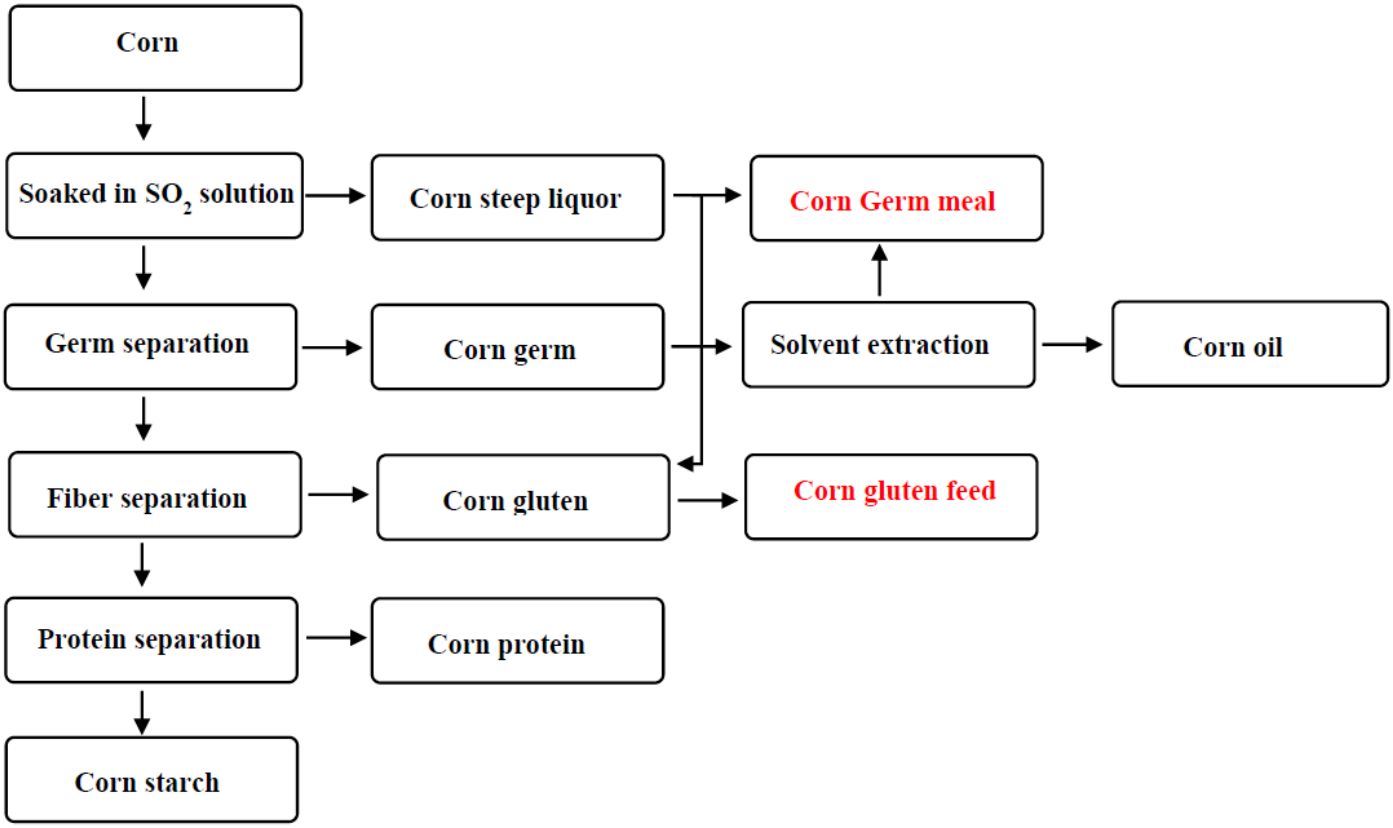

Figure 1

Simplified process of corn wet milling for starch production and its by-products

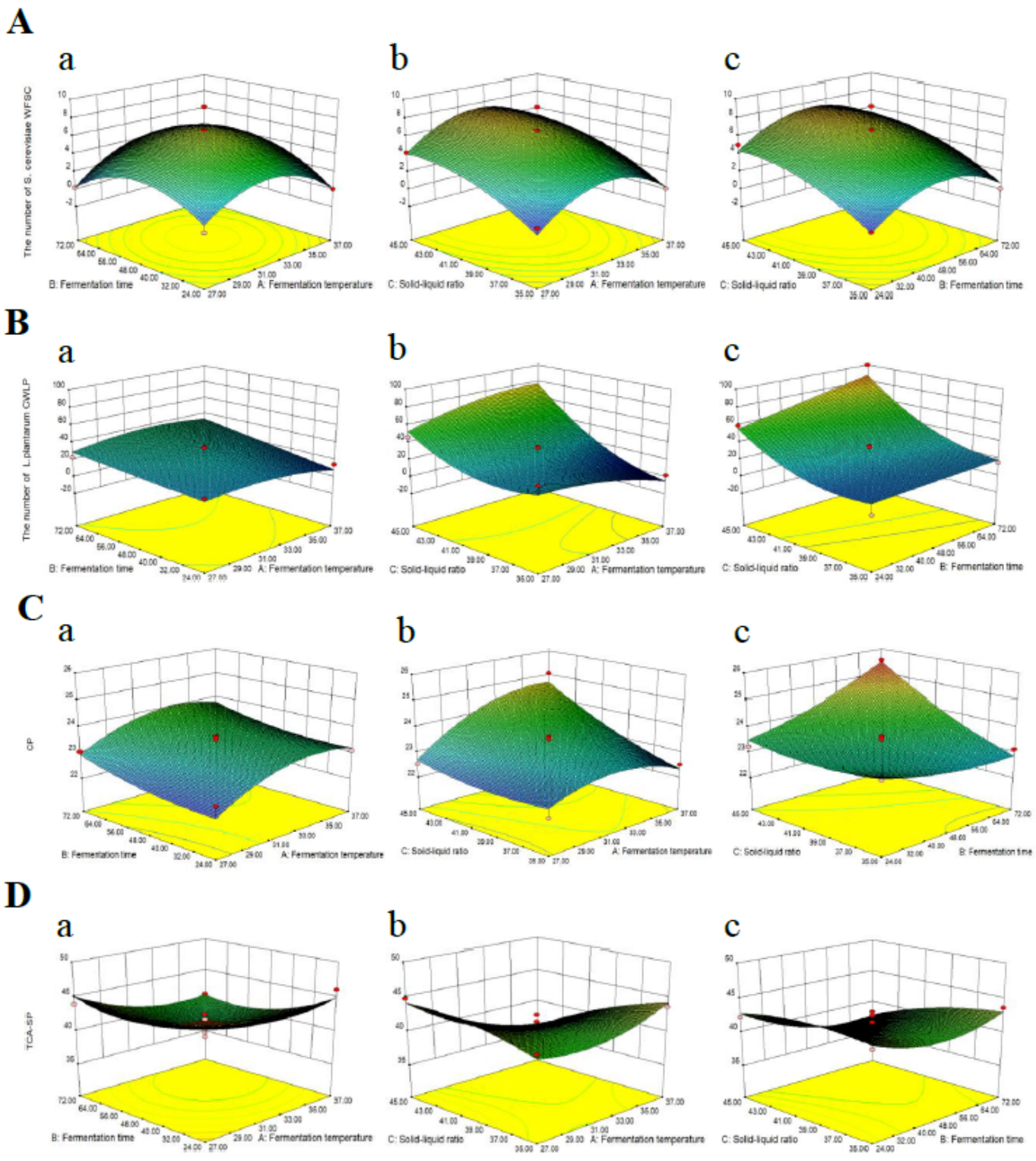

Figure 2

The response surface and contour plots showing the interactive effects of fermentation conditions on the viable count of S. cerevisiae WFSC (A), the viable count of L. plantarum CWLP (B), CP (D) and TCA-SP (D) of CMFSLN (a, fermentation time and fermentation temperature; b, solid-liquid ratio and fermentation temperature; c, solid-liquid ratio and fermentation time)

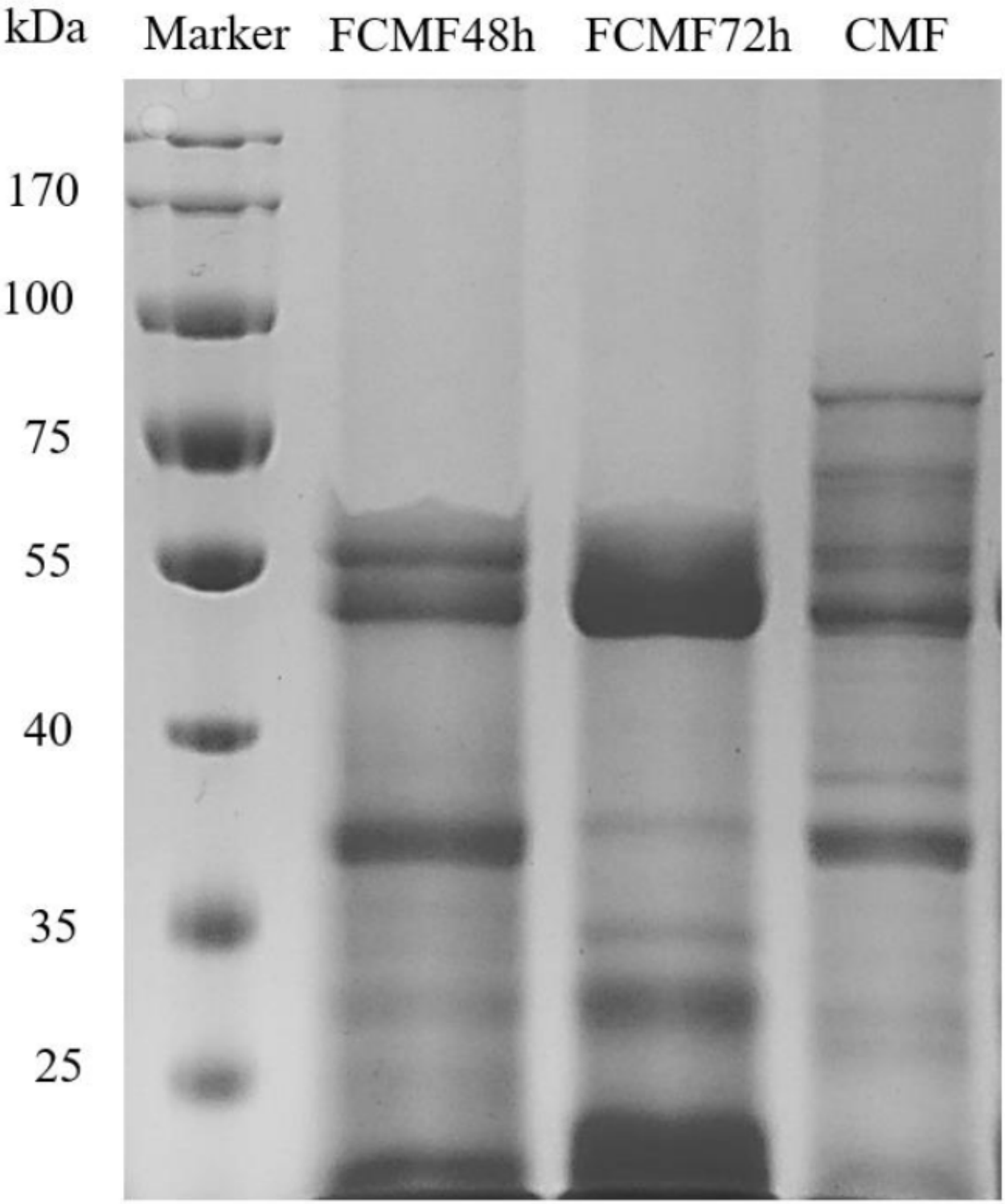

Figure 3

Protein electrophoresis figure analysis of CMF and FCMF (FCMF48h, CMF fermented with S. cerevisiae WFSC, L. plantarum CWLP and neutral protease for 48h, FCMF72h, CMF fermented with S. cerevisiae WFSC, L. plantarum CWLP and neutral protease for 72h).

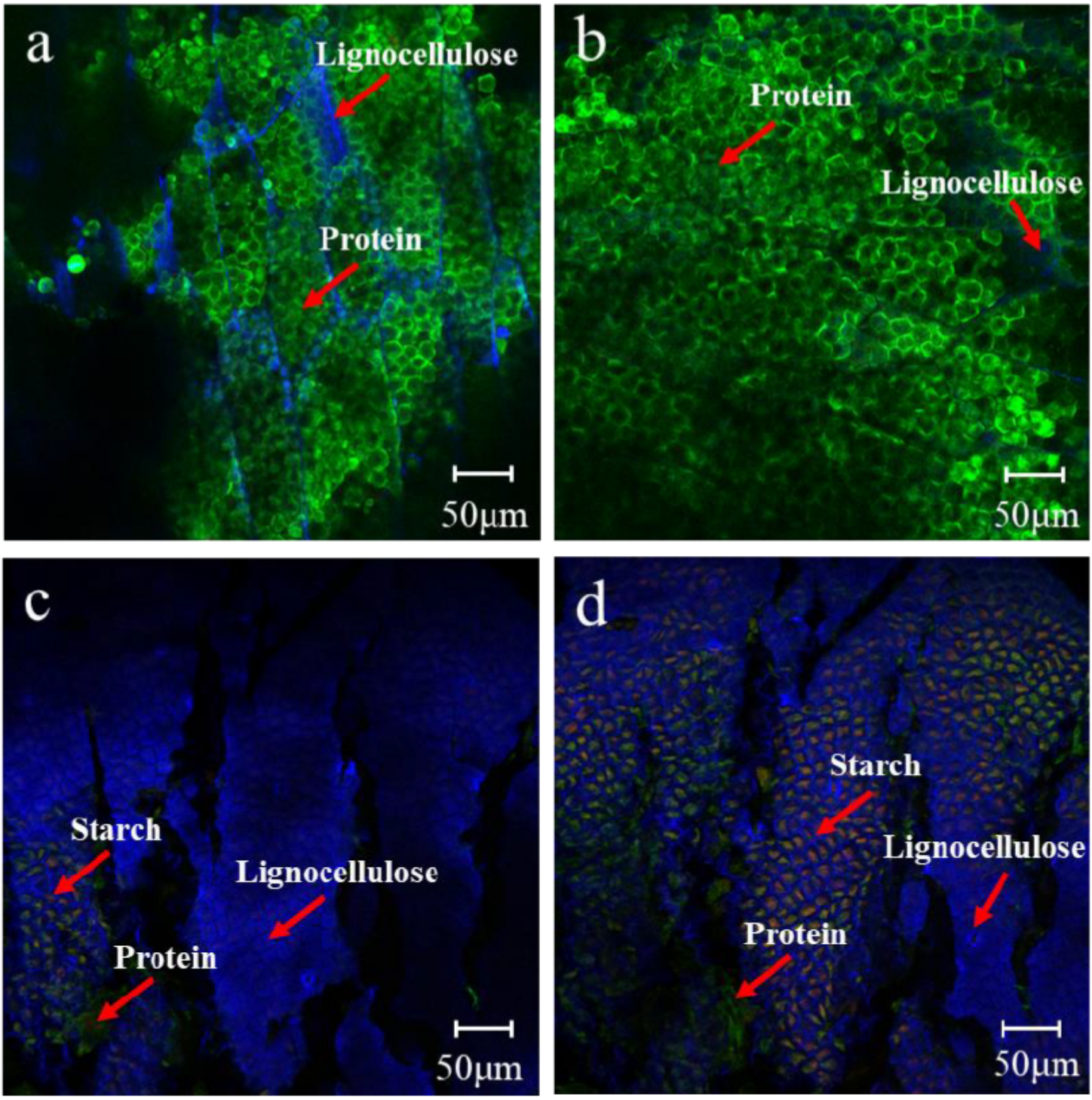

Figure 4

The CLSM images of CMF and FCMF stained with fuorescein isothiocyanate (FITC), calcofuor white (CW) and Concanavalin A (Con A) (a, CGM in unfermented CMF; b, CGM in fermented CMF; c, CGF in unfermented CMF; d, CGF in fermented CMF).