Response surface methodology for the mixed fungal fermentation of *Codonopsis pilosula* straw using *Trichoderma reesei* and *Coprinus comatus*

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Abstract:

The objective of this study was to investigate the cellulose degradation rate (CDR) and lignin degradation rate (LDR) of *Codonopsis pilosula* straw (CPS) and the optimal fermentation parameters for mixed fungal fermentation. Single-factor tests were used to study the effects of the fungal ratio (*Trichoderma reesei*: *Coprinus comatus*), fungal inoculum, corn flour content, and fermentation time on the degradation rate of cellulose and lignin. Based on the results of this experiment, the optimal fermentation factors were identified, and the effects of various factors and their interactions on the degradation rates of cellulose and lignin were further evaluated using the response surface method. The quadratic polynomial mathematical model of degradation rates of the cellulose and lignin in CPS by mixed fungus fermentation was established using Design Expert software v8.0.6. Under the optimal parameters for fungal fermentation of CPS straw (fungal ratio 4:6, fungal inoculum 8%, corn flour content 10%, fermentation time of 15 d), the CDR and LDR reached 13.65% and 10.73%, respectively. Collectively, the mixed fungal fermentation of CPS resulted in decreased lignin and cellulose content, better retention of nutrients, and enhanced fermentation quality. The results of this study

34 indicate that fermentation using *Trichoderma reesei* and *Coprinus comatus* was a productive
35 method for straw degradation, providing a theoretical basis for the development of CPS as feed.
36 **Keywords:** *Codonopsis pilosula* straw, *Trichoderma reesei*, *Coprinus comatus*, lignin
37 degradation rate

INTRODUCTION

39 Lignocellulose is the richest renewable and low-cost biomass energy in the world. Around 10
40 billion tons of lignocellulose is produced annually, about 70% of which is crop straw because of
41 its high yield and considerable resource potential (Sánchez et al., 2008). Despite such an
42 enormous number of straw resources, many developing countries do not maximize the potential
43 of crop straw (Wang et al., 2018). Straw is a very difficult material to break down because of its
44 crystal structure, composed of hemicellulose, lignin, and cellulose. Across the world, large
45 amounts of straw are directly burned, stacked, discarded, or buried, which is a waste of resources
46 and a significant cause of environmental pollution. It is common to use the microbial degradation
47 method on wheat, corn, and other crop straws, but there is little is research on CPS resources
48 (Zhao et al., 2020). With the roots of *Codonopsis pilosula* having a large pharmacological value,
49 the CPS industry is focused on planting, large-scale cultivation, and product development, and
50 does not divert resources to research or the utilization of the large amount of straw resources
51 generated during the production process. Many straw resources are discarded as waste or burned
52 on site, which is both harmful and wasteful, so effective uses for straw resources are needed.

53   *Codonopsis pilosula* is a Chinese herbal medicine that is cultivated worldwide, particularly
54 in China, Japan, and Korea (Chen et al., 2018; Liu et al., 2018). The cultivation area of
55 *Codonopsis pilosula* has significantly increased to match an increase in market demand.
56 Tangshenosid, codonopsine, neochorogenic acid, and lobetyolin are universal active substances
57 in *Codonopsis pilosula* (Gao et al., 2018). These substances have characteristics that have been
58 shown to strengthen the spleen, moisten the lungs, nourish blood, engender liquid, regulate and
59 enhance immune functions, and promote anti-tumor activity (Bai et al., 2018; Zou et al., 2014).
60 Generally, the roots of *Codonopsis pilosula* are used as medicine, but the above-ground portions,
61 including the leaves, stems, and flowers, are discarded (Fu et al., 2018).

62   A previous study reported that the dry matter content of *Codonopsis pilosula* straw (CPS)
63 reached 91.74%, including 17 different amino acids and nitrogen-free extracts (31.63%), which
64 made it a valuable and feasible ruminant feed resource (Ren et al., 2015). Research shows that
65 CPS has low toxicity and can improve animal growth performance, disease resistance, and
66 productivity (Ma et al., 2015). It has also been shown that using CPS as a dietary supplement can
67 increase feed palatability, body resistance, and feed conversion rate, promoting pig growth and
68 fattening, and improving the laying rate of layers (Hong et al., 2019). Lignin is a refractory
69 polymer encasing cellulose and hemicelluloses and impeding their utilization by rumen
70 microorganisms, so CPS has low digestibility and feed values for ruminants (Van Kuijk et al.,
71 2016). Numerous experiments have been conducted to increase the digestibility of crop straw by
72 degrading lignocellulose structure (Zhang et al., 2018), and the pretreatment of high
73 lignocellulose forage using biotechnology has received considerable attention in recent years.

Under specific temperature and humidity conditions, cellulose-degrading lactic acid bacteria and other anaerobic microorganisms degrade the cellulose and hemicellulose in crop straw during anaerobic fermentation (Li et al., 2019; Li et al., 2020). This produces an abundance of acid, requiring the pH value to be further reduced to inhibit the growth of spoilage bacteria (Nayan et al., 2018; Dagar et al., 2015). Applying anaerobic fermentation technology to straw reduces the hemicellulose and cellulose content, improves the digestibility of the straw, prevents a loss of nutrients, improves the fermentation quality and in vitro digestibility, enhances the palatability of straw, and increases its nutritional value (Sun et al., 2019). Diverse biological approaches have been used to study wheat, corn, and other crops, but little research has been conducted on their use in Chinese medicine (Brodeur et al., 2011; Raghuwanshi et al., 2014). The total ban on the use of antibiotics as growth promoters in animals was introduced in the European Union on 1 January 2006 (Regulation [EC] No, 1831/2003; Anna et al., 2021). This led to a gradual, worldwide ban on antibiotic feed additives to ensure the healthy and sustainable development of animal husbandry. China banned antibiotics as a growth promoter and prohibited the prophylactic use of antibiotics in feed in 2020 (except in traditional Chinese medicine). Therefore, developing a green, safe, and healthy antibiotic alternative with no side effects has become an important issue in feed production. Chinese medicine is considered one of the potential alternatives to antibiotics. Using the non-medicinal, unused plant parts from the production process of traditional Chinese medicine resources as raw feed materials to develop new feeds would save straw resources from being wasted and help meet the increasing demand for animal feed products. This study used lignocellulose-degrading bacteria, such as mold and white rot fungi, to ferment and prepare high-quality fermented forage (Zhao et al., 2020; Sánchez et al., 2009; Kogo et al., 2017). Cellulase and laccase were produced by *Trichoderma reesei* and *Codonopsis pilosula*, which can break down lignocellulose in CPS. *Trichoderma reesei* and *Codonopsis pilosula* are reported to grow synergistically, increasing enzyme production efficiency (Ge et al., 2009).

The objective of the present study was to determine if CPS was able to be developed as feed. This study tested the following hypotheses: (i) The lignocellulose in CPS could be degraded by *Trichoderma reesei* and *Coprinus comatus* and (ii) the optimal CPS fermentation conditions could be identified using response surface methodology (RSM), providing guidance for preparing high-quality CPS-fermented feed.

MATERIALS AND METHODS

Test materials

Fungal strains and spawn preparation

The *Trichoderma reesei* (strain CGMCC 3.5218) and *Coprinus comatus* (strain CC900) used in this study were purchased from the China General Microbiological Culture Collection Center (Beijing, China) and the Tianda Institute of Edible Fungi (Jiangsu, China), respectively. Before beginning the experiment, two strains of fungi were resuscitated according to the protocols of

112 our research team. Two fungi were maintained on potato dextrose agar plates containing (g/L):
113 potato 200, dextrose 20, and agar 15, and the pH value was not adjusted. The fungi were grown
114 in a 28 °C constant temperature incubator for roughly a week until mycelia colonized and
115 completely covered the agar plates. Finally, the fungi were stored at 4 °C and subcultured every
116 3 months.

117 *Codonopsis pilosula* straw

118 CPS was collected at the ripening stage (dry matter, 90%) from Dingxi City, Gansu Province
119 (34.26–35.35 N, 103.52–105.13 E). The collected CPS was then chopped into approximate
120 lengths of 2–3 cm and then crushed into granules with an average particle size of 0.2 cm using a
121 pulverizer (93ZR-8.0C, Shengtailong Mechanical Equipment Company, Feicheng, China) to
122 facilitate subsequent fermentation. These granules were stored in sealed plastic bags in the
123 laboratory and kept in a cool, dry room to avoid deterioration and rotting. The chemical
124 compositions of CPS are presented in Table 1.

125 Preparation of liquid fermentation broth

126 The liquid fermentation medium was improved and prepared based on the methods described by
127 Kogo et al. (2017) using S. *pastorianus*. The medium was composed of 200 g/L potatoes, 20 g/L
128 glucose, 2 g/L anhydrous ammonium sulfate, 1 g/L peptone, 1 g/L anhydrous potassium
129 dihydrogen phosphate, and 1 g/L anhydrous magnesium sulfate, which was cultured for 2 d to
130 *Trichoderma reesei* and 5 d to *Coprinus comatus* in a rotary shaker (150 rpm) at 28 °C. To
131 guarantee that the spore concentration was constant in this investigation (1×10^7 cells/mL), the
132 hemocytometer plate was used to count the spore concentrations of the two strains after culture
133 (Chen et al., 2018; Chen et al., 2011; Xu et al., 2017).

134 Experimental design for *Codonopsis pilosula* straw fermentation

135 Substrate preparation

136 The substrate included 90% CPS and 10 % corn flour. The substrate and water were well mixed
137 in a 2:3 weight basis ratio and compacted into self-sealing bags. (17 cm × 25 cm).

138 Single-factor inoculation of *Trichoderma reesei* and *Coprinus comatus*

139 Using fungus ratio (*Trichodemma reesei*: *Coprinus comatus*), mixed fungal inoculum, corn flour
140 content, and fermentation time as single factors, the effects on the degradation rate of lignin and
141 cellulose in the CPS were investigated, with three replicates in each group.
142   Group 1: Testing for best fungal ratio: the fungal liquid was inoculated with *Trichodemma*
143 *rcesei* and *Coprinws comatus* with ratios of 10:0, 7:3, 5:5, 3:7, and 0:10 in the substrate. The
144 mixed fungal inoculum was 10% (water content basis), corn flour content was 10% (weight
145 basis), and fermentation time was 10 d.

146   Group 2: Testing for best fungal inoculum: mixed fungal inoculums of 5 %, 10 %, 15 %,
147   and 20 % (water content basis) were added to the substrate, using the optimal fungal ratio
148   determined from Group 1 and same proportions and fermentation time as Group 1.
149   Group 3: Testing for optimal nutrient additive amount: 5 %, 10%, 15 %, and 20 % (weight
150   basis) of corn flour was added into the substrate, using the optimal fungal ratio determined in
151   Group 1, the optimal fungal inoculum determined from Group 2, and a 10-day fermentation time.
152   Group 4: Testing for optimal fermentation time: Using the optimal fungal ratio, fungal
153   inoculum, and nutrient additive amounts determined in Groups 1-3, the fermentation was carried
154   out on the substrate for 5 d, 10 d, 15 d, and 20 d.

Conditional optimization of fermentation

156   The effects of numerous parameters and their interactions on the breakdown of cellulose and
157   lignin in corn straw were evaluated using the response surface approach. According to the design
158   principle of the Box-Behnken experiment, four factors were selected, including fungal ratio,
159   mixed fungal inoculum, corn flour content, and fermentation time, while CDR and LDR were
160   taken as response values (Design Expert 8.0.6). An experimental response surface analysis was
161   conducted with three levels of each factor. A mathematical model was established between the
162   degradation rate of lignin and cellulose and the various factors tested and these calculations were
163   used to determine the optimal conditions for using *Trichoderma reesei* and *Coprinus comatus*
164   mixed fermentation to break down lignocelluloses (supplemently table 1).

Characterization of *Codonopsis pilosula* straw

166   Characterization scanning electron microscope (SEM) imaging was carried out using a Gemini
167   500 SEM. The chemical phase analysis was carried out using a PANalytical X-ray diffractometer
168   (XRD), which was equipped with CuKα radiation (λ = 0.15406 nm). Fourier-transformed
169   infrared (FTIR) spectra were obtained from an FTIR spectrometer (FTIR-7600, Lambda
170   Scientific, Australia).

Chemical analyses

172   Each sample's neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin were
173   measured after the single-factor test. Dry matter (DM), crude protein (CP), ether extract (EE),
174   ash, NDF, ADF, lignin, and volatile fatty acids were evaluated in the samples before and after
175   response surface methodology optimization. After drying the samples to a constant weight at 65
176   °C, the DM content of the samples was calculated. CP content was calculated by multiplying
177   nitrogen content by 6.25. The diethyl ether extract was determined using the Soxhlet method
178   (Wang et al., 2021). After the samples were heated to 600 °C for six hours in a muffle furnace,
179   the amount of ash was measured. The ash-free NDF (VanSoest et al., 1991), ash-free ADF, and
180   lignin were determined as previously described. We then calculated the difference between NDF
181   and ADF to determine the hemicellulose content, and the difference between ADF and acid
182   detergent lignin (ADL) to determine the cellulose content (VanSoest., 1973). A total of 20g of
183   uniformly mixed fermented feed was placed in a juicer, and 180 mL of distilled water was added,

184 homogenized for 2min, and filtered with four layers of gauze to prepare a crude extract. This was
185 then filtered with double-layer filter paper and left standing for 0.5 h. Analysis of organic acids
186 was carried out by high-performance liquid chromatography (HPLC). After adequate dilution,
187 the samples were filtered through a 0.2 μm polypropylene filter before analysis in a Dionex
188 UltiMate 3000 RSLC HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) connected
189 to a Shodex RI-101 differential refractive index detector (Showa Denko, Tokyo, Japan). The
190 organic acids were separated using the Aminex HPX-87H analytical column coupled to a guard
191 column (Bio-Rad, Hercules, CA, USA). A series of standard solutions, including acetic acid,
192 lactic acid, butyric acid, and propionic acid, were prepared and analyzed together with the
193 samples. The temperature was set to 30 ℃ and the mobile phase consisted of a methanol solution
194 of 10% and 0.1% v/v phosphoric acid solution of 90% with a flow rate of 0.6 ml/min (Huang et
195 al., 2021).

196 **Statistical analyses**

197 Experimental data, including the chemical compositions of CPS after different treatments, were
198 expressed as means and analyzed by IBM's Statistical Product and Service Solutions software
199 (SPSS 21.0). ANOVA was conducted to evaluate differences, followed by a Duncan's multiple
200 range test. Differences were considered statistically significant at $P < 0.05$.
201

202 **RESULTS**

203 **Effect of single-factor inoculation of *Trichoderma reesei* and *Coprinus comatus***

204 **on the degradation rate of cellulose and lignin in *codonopsis pilosula* straw.**

205 **Influence of the fungal combination ratio**

206 As the proportion of *Trichoderma reesei* gradually decreased and *Coprinus comatus* gradually
207 increased (Fig. 1a), cellulose and lignin also decreased gradually. Using a 3:7 ratio (*Trichoderma*
208 *reesei*: *Coprinus comatus*), the CDR and LDR reached 12.3 % and 5.67 %, respectively, with an
209 initial transient increase followed by a slight decrease in degradation rate, verifying that
210 *Coprinus comatus* is important to lignin decomposition (Fig. 1b). When *Trichoderma reesei* was
211 not included in the substrate (0:10 ratio), the CDR decreased.

212 **Influence of fungal inoculum**

213 The degradation rates of cellulose and lignin increased as the inoculum size increased (Fig. 2a).
214 The peak CDR (13.22 %) and LDR (8.44 %) were observed using an inoculum size of 10 % (Fig.
215 2b).

216 **Influence of cornmeal amounts**

217 At a corn flour concentration of 10 %, the cellulose and lignin content was the lowest, and peak
218 CDR (13.30 %) and LDR (10.47 %) were observed (Figs. 3a and 3b). The degradation then fell,
219 marginally, as the corn flour concentration increased ($P < 0.05$). Batch fermentation was
220 conducted to save costs, and the optimal addition of corn flour was set to 10 %.

Influence of fermentation duration

222 *Trichoderma reesei* and *Coprinus comatus* primarily grew hyphae in the first 5 d, but the CDR
223 and LDR increased significantly over time. The degradation rates of cellulose and lignin
224 decreased after 10 d of fermentation, and after 15 d, the CDR and LDR were 13.61% and 9.33%,
225 respectively (Figs. 4a and 4b), indicating the optimal fermentation duration was 15 d.

Optimization of fermentation conditions of *Trichoderma reesei* and *Coprinus*

comatus using response surface methodology

Response surface experimental design and result analysis

229 Design Expert software was used to further optimize the parameters of each factor. A, C, D, AB,
230 AC, CD, A^2, B^2, C^2, and D^2 had highly significant effects on the CDR ($P < 0.01$), and C and AD
231 had significant effects on the CDR ($P < 0.05$), indicating that these were solid (supplemently
232 table 2). No significance was found for the missing items, indicating that there were no abnormal
233 points in the test data, and that the model was appropriate (supplemently table 3). Moreover, A,
234 B, C, D, AB, BC, B^2, C^2, and D^2 had highly significant effects on LDR ($P < 0.01$), which
235 indicates that these were important factors in the solid-state fermentation process. The missing
236 items were not significant, indicating that there were no abnormal points in the test data and that
237 the model was appropriate (supplemently table 4).
238 According to the regression analysis of the response surface coefficient of CDR, the fitting
239 equation of the model was: Y= 9.32A + 3.66B + 5.45C + 11.91D－0.95AB + 0.56AC + 0.47AD
240 + 0.07BC－0.04BD + 0.39CD－1.08A2 -0.32B2－0.37. The regression model was highly
241 significant ($P < 0.01$), while the lack-of-fit test was not significant ($P = 0.8196$), indicating that
242 the regression equation had a good fitting degree. The regression equation's coefficient of
243 determination (R^2) was 0.93, indicating that this model can explain 93% of the variation in CDR,
244 suggesting that it is a good fit for actual data (supplemently table 3).
245 According to the regression analysis of the response surface coefficient of LDR, the fitting
246 equation of the model was: Y= 4.25A + 8.59B－0.46C + 5.54D－0.95AB－0.17AC + 0.11AD +
247 0.30BC + 0.04BD + 0.15CD－0.08A2－0.24B2. Consistently, the regression model was highly
248 significant ($P < 0.01$), and the lack-of-fit test was not significant ($P = 0.2428$), which indicates
249 that the regression equation had a good fitting degree. The R^2 of the regression equation was 0.93,
250 indicating that 93% of the variation in CDR could be explained by this model and that the model
251 is a good fit for actual data (supplemently table 4).

Response surface analysis of cellulose degradation rate

253 Based on the regression equation, the response surface plot was generated to directly reflect the
254 influence of each element and the interaction of two factors on the response value.
255   The steeper the surface slope in the interactive graph, the closer the contour line was to the
256 ellipse, suggesting greater influence of this factor on the response value. Two factors were fixed,
257 and the interaction between the other two factors was analyzed. When the fungal ratio increased,
258 and the fungal inoculum decreased, the CDR increased, and the trend slope on the cross plot was
259 high, indicating that these two factors significantly influenced the CDR (supplemently Fig. 1b).
260 The cellulose degradation rate exhibited an upward trend with an increase in fungal ratio and
261 corn flour content (supplemently Fig. 2b). With an increased fungal ratio and fermentation time,
262 the CDR also increased, and the slope of the interaction graph between them was high, indicating
263 a significant influence on the CDR (supplemently Fig. 3b). The contour lines of the above
264 interaction diagrams were all elliptical, indicating significant interactions, consistent with the
265 results of the variance analysis (supplemently Fig. 4b).

Response surface analysis of lignin degradation rate

267 With increased fungal ratio and inoculum size, the LDR exhibited a downward trend, indicating
268 that both significantly influenced the LDR (supplemently Fig. 5b). The LDR decreased with
269 increased inoculum and exhibited a transient increase followed by a decrease as the amount of
270 corn flour added increased (supplemently Fig. 6b). The contour lines of the above interaction
271 diagrams were all elliptical, indicating significant interactions, consistent with the variance
272 analysis results.

Response surface optimization results and model verification

274 The optimal conditions for the simultaneous degradation of cellulose and lignin, obtained using
275 Design Expert software, were as follows: a fungal ratio of 4:6, inoculum size of 8%, a corn flour
276 content amount of 10%, and a fermentation duration of 15 d. Under these conditions, the
277 predicted CDR and LDR were 14.37% and 11.65%, respectively. The optimal conditions of
278 model optimization were tested to verify the model's effectiveness. The solid-state fermentation
279 test was carried out, and the verification test was set up with three replicates, with the average
280 degradation rate of cellulose and lignin being 13.65% and 10.73%, respectively. These results
281 indicate that this model can predict the degradation of cellulose and lignin. Other chemical
282 components of fermented CPS were then determined using this model. The CP increased
283 significantly, ADF, NDF, cellulose, and lignin decreased significantly, while crude ash and CP
284 did not change significantly (Table 2).

Fermented *Codonopsis pilosula* straw was characterized through XRD, FTIR,

SEM, and XRD analyses of *Codonopsis pilosula* straw

287 The crystallinity of cellulose in *Codonopsis pilosula* straw stem before and after fermentation
288 was analyzed in an XRD experiment, as shown in Fig 5. The two well-defined crystalline peaks
289 represent amorphous at around $2\theta = 22°$ and crystalline peaks $2\theta = 27°$. The peak values of

290 crystal planes 2θ = 22° and 2θ = 27° in the fermentation treatment group were significantly lower
291 than those in the control group, indicating that the crystallinity of the straw after treatment with
292 *Trichoderma reesei* and *Coprinus comatus* was lower than that in the control group. As expected,
293 the magnitude of these crystalline peaks reduced upon hydrolysis, which successfully eliminated
294 the non-cellulosic materials in the amorphous regions of the *Codonopsis pilosula* straw stem.
295 The fermentation treatment increased the amount of cellulose exposed on the fiber surface,
296 thereby increasing surface roughness.

FTIR analysis of *Codonopsis pilosula* straw

298 To further investigate the influence of *Trichoderma reesei* and *Coprinus comatus* on the
299 structure of *Codonopsis pilosula* straw, their chemical structures were characterized using a
300 Fourier-transformed infrared (FTIR) spectrometer (Fig. 6). Fig. 6 shows that, compared with the
301 untreated *Codonopsis pilosula* straw, the absorption of the fermented *Codonopsis pilosula* straw
302 by *Trichoderma reesei* and *Coprinus comatus* decreased at 1650 and 1429 cm^{-1} (the
303 characteristic absorption peak of benzene ring), indicating that lignin was degraded to a certain
304 extent. The peak of 1201-1357cm^{-1} (C-O stretching vibration in lignin) represents the byproducts
305 of lignin decomposition, such as phenol, ether, alcohol, and ester. The increase in the intensity of
306 these peaks indicates an increase in these byproducts. After pretreatment, the peak at 1022cm^{-1}
307 (C-O stretching vibration, carbohydrate [cellulose, hemicellulose] or polysaccharide) was
308 enhanced, showing that cellulose is degraded into sugars. The 3300-3500cm^{-1} wide absorption
309 band corresponds to carbohydrates (cellulose, hemicellulose, starch, monosaccharide, etc.). The
310 decrease of the peak here indicates that the stretching vibration of the hydroxyl group was
311 strengthened. This may be because the ether bond broke, making the molecular structure looser,
312 exposing the internal hydroxyl group, and increasing the hydroxyl group of lignin. This shows
313 that the lignin undergoes a demethylation reaction, which can reduce the steric hindrance inside
314 the lignin and improve its reaction activity. These results show that the chemical structure of the
315 cellulose and lignin of the *Codonopsis pilosula* straw were destroyed by the introduced strains,
316 effectively degrading the cellulose and lignin.

SEM analysis of *Codonopsis pilosula* straw

318 The morphological structures of both raw and fermented *Codonopsis pilosula* straw samples
319 were examined through a scanning electron microscope (SEM) and are presented in Fig. 7. The
320 surface of the straw in the control group, as shown in Fig. 7, is relatively smooth, covered with
321 amorphous components, and has a relatively complete structure. After adding two kinds of
322 *Trichoderma reesei* and drumstick fungi for fermentation, the straw surface showed corrosion
323 and tearing, with the straw surface becoming loose, with exposed cellulose and lignin, and
324 broken and rough fiber bundles. Lignocellulose-degrading enzymes secreted by *Trichoderma*
325 *reesei* and *Coprinus comatus* during the growth process led to some degradation in *Codonopsis*
326 *pilosula* straw. With the shedding of the primary cell wall, more inferior walls are exposed,
327 which is conducive to the in-depth role of enzyme molecules.

DISCUSSION

329 **Effect of *Trichoderma reesei* and *Coprinus comatus* on the degradation rate of**

330 **cellulose and lignin in *Codonopsis pilosula* straw.**

331 *Codonopsis pilosula* is a traditional medicine that has been used worldwide for many decades
332 (Gao et al., 2018). Nevertheless, *Codonopsis pilosula* straw (CPS) has been largely understudied
333 and is often burnt as waste. Few studies have examined the use of CPS as crop straw feed. It has
334 been established that straw is primarily made up of cellulose, hemicellulose, and lignin.
335 Biodelignification can be done using microbes, as these microorganisms produce enzymes to
336 attack, and degrade polymers in lignocellulosic substrates. Many microorganisms in nature can
337 degrade lignin, including fungi and bacteria. This study used the microbial fermentation method
338 to ferment and degrade the lignocellulose of CPS, specifically using the fungal microorganisms
339 *Trichoderma reesei* and *Coprinus comatus*. Genetically-engineered *Trichoderma reesei* is one of
340 the most promising fungi for cellulase production, with an estimated 60 to 100 g of extracellular
341 protein per liter of cultured *Trichoderma reesei* (Ike et al., 2010). *Coprinus comatus* reportedly
342 produces ligninolytic enzymes, which delignify and break down the recalcitrant components in
343 plant cell walls. White-rot fungi have been reported to be the most effective microorganisms for
344 decomposing lignin (Ahmed et al., 2013), as the basidiomycetes of white-rot fungi can efficiently
345 degrade lignin (Wong et al., 2009) by producing various extracellular ligninolytic enzymes, such
346 as laccase, manganese peroxidase, and lignin peroxidase (Nagai et al., 2007). Lignin-modifying
347 enzymes play a critical role in degrading lignin compounds for feed suitable for ruminants
348 (Andrew et al., 2016). Overwhelming evidence indicates that the combination of white rot fungi
349 with other fungi significantly improves straw fiber degradation. In one study, wheat straw was
350 treated with a variety of white rot fungi to improve the degradation rate of cellulose and lignin
351 (Arora et al., 2011). In this study the average degradation rate of cellulose and lignin in CPS
352 were 13.89% and 11.18%, respectively, with microbial fermentation treatment. In a separate
353 study, oat straw degradation rates of cellulose and lignin reached 75% and 55%, respectively,
354 when white rot fungi and filamentous fungi were mixed (Stepanova et al., 2003). *Schizophyllum*
355 *commune* has demonstrated degradation rates of 30-40% in finger millet straw, 27-32% in paddy
356 straw, 21% in wheat straw, and 26% in maize straw (Kumar et al., 2022). Moreover,
357 carboxymethylcellulose activity was significantly enhanced when using potato peel residue as
358 the substrate and treating the mixture with *Aspergillus Niger* and *Trichoderma* (Taher et al.,
359 2016).

360 Using a comprehensive analysis of SEM, XRD, and FTIR, the degradation degree of
361 lignocellulose by *Trichoderma reesei* and *Coprinus comatus* was analyzed at the microscopic
362 level to comprehensively evaluate the effectiveness and feasibility of the fermentation method.
363 The results show that this method can effectively degrade the lignocellulose of CPS. A previous
364 study found, using SEM, that the process of bacteria acting on wood can be divided into three
365 processes: erosion, ditching, and drilling (Rihani et al., 2001). Bacteria first grow to the middle
366 thin layer of wood cells, causing the erosion of fiber walls, and then grow, creating cavities, or
367 ditches, in the cell wall. Another study used XRD to confirm that the relative crystallinity of corn

368 cobs, corn stalks, and sorghum stalks increased after ammonia was combined with ultrasonic
369 pretreatment, likely because the pretreatment destroyed the amorphous region of lignocellulosic
370 raw materials and exposed the crystalline region (Xu et al., 2017). Zhang et al. used FTIR to
371 determine the changes to chemical groups in poplar after pretreatment with white rot fungi
372 combined with a NaOH solution (Zhang et al., 2016), and Xu et al. used SEM, XRD, and FTIR
373 to evaluate the pretreatment effect of sodium lignosulfonate on corn stalks (Xu et al., 2015). Ye
374 et al. also used SEM, XRD, and FTIR to characterize the microstructure changes of paulownia
375 after physical/chemical pretreatment (Ye et al., 2016). In this study, strains producing cellulase
376 (*Trichoderma reesei*) and laccase (*Coprinus comatus*) were used to degrade CPS, and the
377 degradation effect of CPS was evaluated through various fermentation conditions. The results
378 showed that these strains had a degradation effect on CPS lignocellulose.

379 The growth of microorganisms is influenced by several factors, including: moisture,
380 inoculum amount, nitrogen source, carbon source, nutrient requirements, and fermentation time.
381 Other studies have assessed the effects of pH, carbohydrates, and nitrogen source ratios in the
382 straw fermentation process and found that adding a suitable amount of carbs and nitrogen
383 sources during the process can significantly improve the feed fermentation quality (Tao et al.,
384 2017). This study demonstrated that fungal ratio, fungal inoculum, corn flour content, and
385 fermentation duration had important effects on the fermentation process of CPS. Increased
386 *Coprinus comatus* led to increased lignin degradation and a synergistic degradation of cellulose.
387 This finding explains the increase in the cellulose breakdown rate when the fungal ratio
388 increased and the concentration of *Trichoderma reesei* decreased. When the inoculum was too
389 large, the microorganisms grew too quickly; the insufficiently dissolved oxygen in the
390 fermentation system reduced microorganism activity and enzyme production rate. In contrast,
391 when the inoculum was too small, microorganism growth and the enzyme production rate were
392 slow. Carbon and nitrogen supplies are reportedly significant factors for microbial lignin
393 degradation and enzyme production and modifying the culture conditions can greatly increase
394 the production of lignin-degrading enzymes (Leontievsky et al., 1994). Different proportions of
395 cornmeal, used as a source of carbon and nitrogen, were tested in this study to examine their
396 effects on CPS fermentation, and 10% cornmeal yielded optimal results. Fermentation duration
397 also impacts CPS fermentation results. When fermentation time is too short, the bacteria cannot
398 produce the enzymes that will degrade the straw lignocelluloses, but a fermentation time that is
399 too long can impact the quality of the fermentation (Zhao et al., 2020).

400 A symbiosis of microorganisms can increase the palatability and digestibility of straw for
401 livestock and poultry, reducing feed rate, costs, and environmental pollution. Research shows
402 that fermentation with lactic acid bacteria, yeast, and *Bacillus amyloliquefaciens* increases CP
403 content in rape straw (Tuyen et al., 2013). In this study, *Trichoderma reesei* and *Coprinus
404 comatus* were added to CPS at a 4:6 ratio, leading to different degrees of cellulose and lignin
405 degradation the straw. More lignin was destroyed by more *Coprinus comatus*, explaining the
406 increase in cellulose breakdown when the relative concentration of *Trichoderma reesei* declined.
407 It is widely acknowledged that *Trichoderma reesei* and *Coprinus comatus* can synthesize laccase,
408 manganese peroxidase, lignin peroxidase, and cellulase to degrade cellulose and lignin as quickly

409 as possible in the solid-state fermentation process with the right inoculation dosage. However, if
410 the inoculum is too high, the nutrients in the solid-state fermentation medium are consumed too
411 quickly, affecting the growth of the fungal strain later in the fermentation process. At the same
412 time, the metabolites produced increase in a short time, reducing the synthesis rate of various
413 decomposing enzymes. The benefit of mixed fungal fermentation is that different strains exhibit
414 different enzyme-producing properties and complement one another, resulting in the rapid
415 breakdown of lignocelluloses in straw (Gutierrez-Correa et al., 1999). Laccase activity
416 significantly improved when cultured Trametes Versicolor and oyster mushrooms were mixed
417 with other microorganisms such as fungi, indicating that mixed cultures can achieve cellulose
418 and lignin degradation by strengthening the extracellular lignocellulose-degrading enzymes as
419 well as improving other active factors (Petr., 2004). *Trichoderma reesei* and *Coprinus comatus*
420 were used to ferment CPS in this experiment, and the impact on cellulose degradation was the
421 most significant after a fermentation time of 15 days. As the fermentation time increased, the
422 remaining nutrients in the solid fermentation medium gradually decreased, the growth and
423 metabolism of the strains slowed, and the secretion of related enzymes used for degradation
424 decreased, resulting in a gradual decrease in the rate of cellulose and lignin degradation.

Conclusions

426 The optimal fermentation conditions of Trichodemma reesei and Coprinus comatus for lignin
427 degradation in CPS were obtained. The fungal combination fermentation could significantly
428 increase cellulose, hemicellulose, and lignin degradation rates for converting CPS to more edible
429 feedstuffs.

430

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432 Conceptualization, Penghui Guo.

433 Experiment, Ti Wei, Hongfu Chen, Dengyu Wu.

434 Data curation, formal analysis, software, and writing-original draft, Ti Wei, Hongfu Chen,
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436 Resources, and writing-re*view and editing, Dandan Gao, Yong Cai, Xin Cao, Hongwei* Xu,
437 Julian Yang, Penghui Guo.

438 All authors have read and agreed to the published version of the manuscript. By successfully
439 converting *Codonopsis pilosula* straw into a new type of ruminant fermentation feed, we will
440 promote the improvement of the ecological environment and the cyclic development of the
441 sector

442 **Funding:** The Fundamental Research Funds for the Central Universities of Northwest Minzu
443 University (31920220038, 31920220025, 31920230027); Science and Technology Project of
444 Gansu Province (21JR1RA218); Longyuan Youth Innovation and Entrepreneurship Talent
445 Project of Gansu Province (2021LQGR36); Research on key technologies of efficient and
446 healthy beef cattle breeding and comprehensive development and utilization of forage resources
447 (LYLK201008).

448 **Institutional Review Board Statement**: Not applicable.

449 **Informed Consent Statement**: Not applicable.

450 **Data Availability Statement**: The data presented in this study are available in the article.

451 **Conflicts of Interest**: The authors declare no conflict of interest.

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643

Figure 1

Figure.1 Effects of different fungal ratios on the degradation of cellulose and lignin in *Codonopsis pilosula* straw, (a) Cellulose and lignin content, (b) cellulose and lignin degradation rate
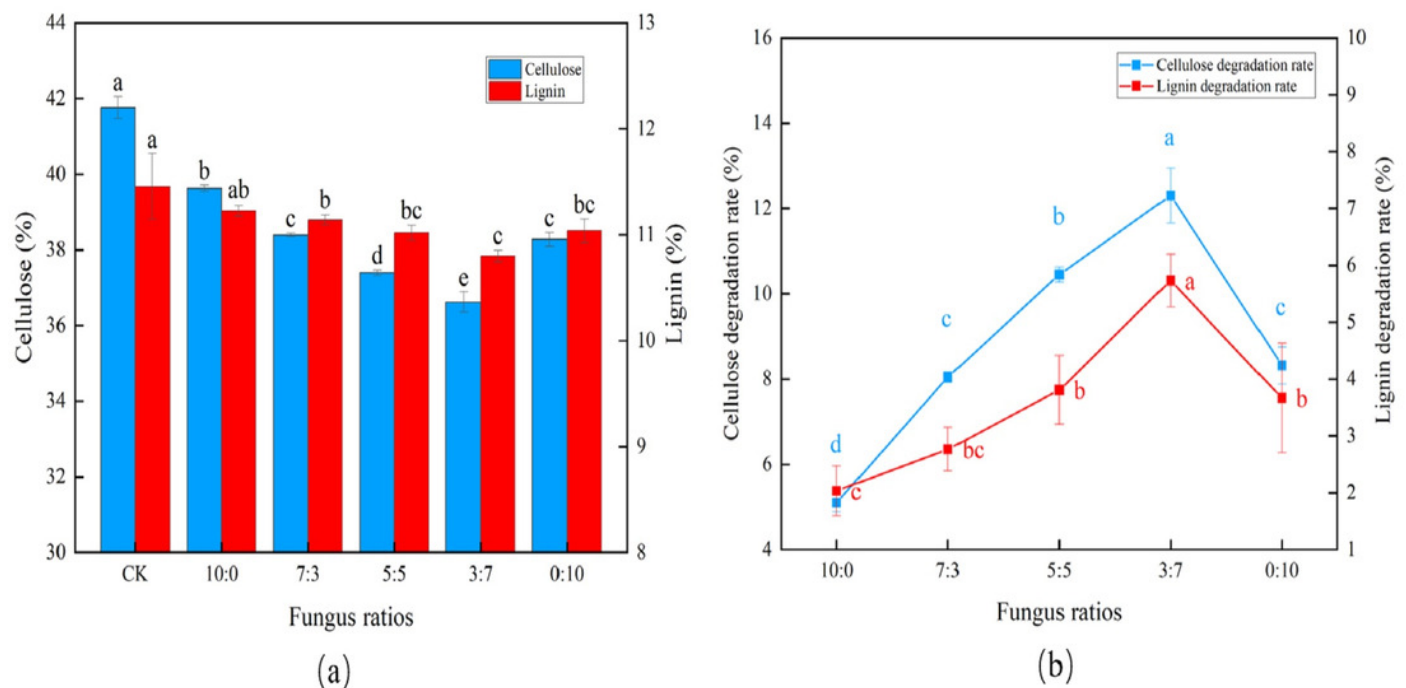
Figure 2

Figure.2 Effects of different inoculum on the degradation of cellulose and lignin in *Codonopsis pilosula* straw, (a) Cellulose and lignin content, (b) cellulose and lignin degradation rate
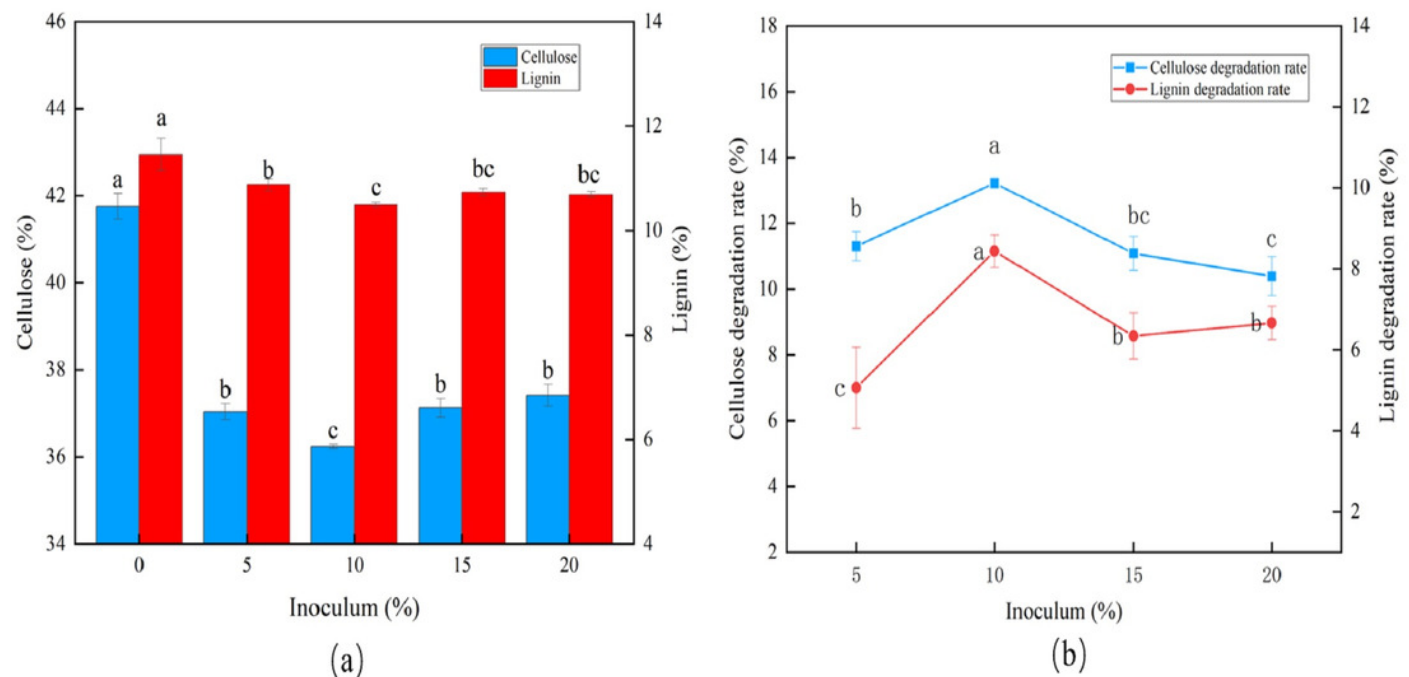
Figure 3

Figure.3 Effects of different corn flour amounts on the degradation of cellulose and lignin in *Codonopsis pilosula* straw, (a) Cellulose and lignin content, (b) cellulose and lignin degradation rate
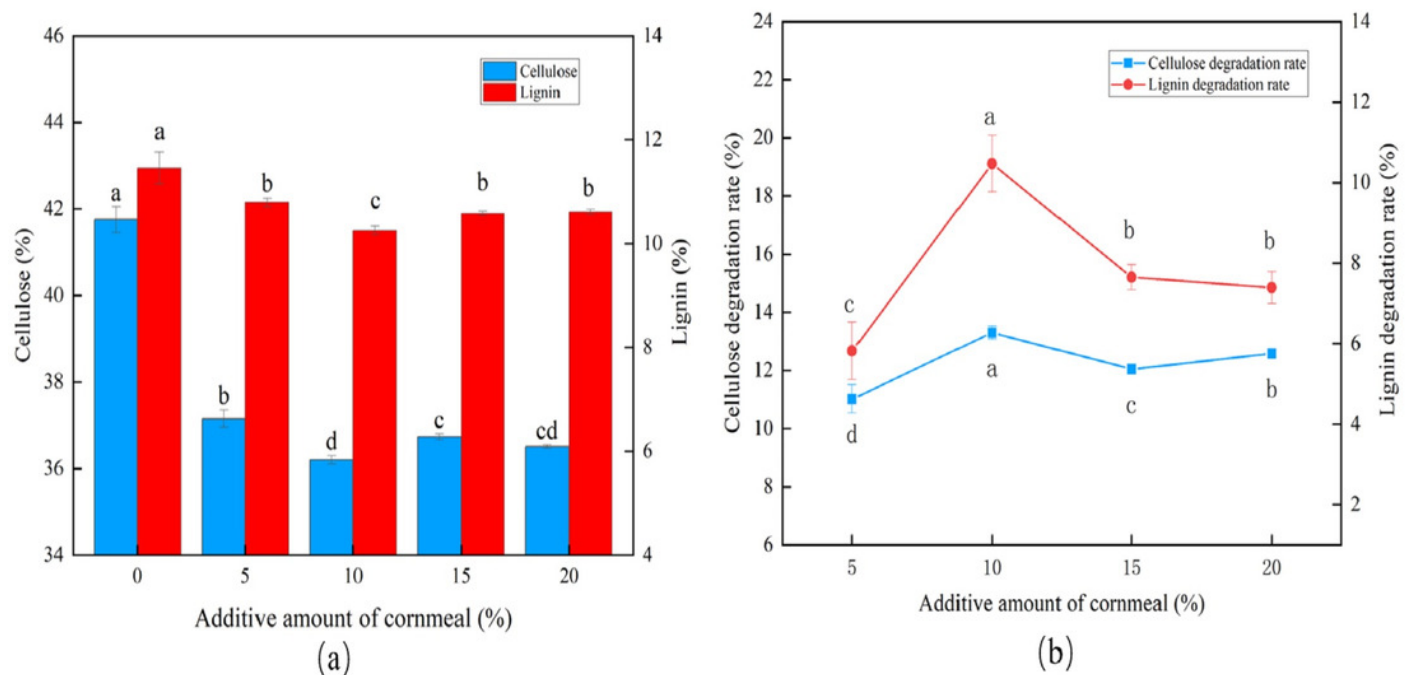
Figure 4

Figure.4 Effects of different fermentation times on the degradation of cellulose and lignin in *Codonopsis pilosula* straw, (a) Cellulose and lignin content, (b) cellulose and lignin degradation rate
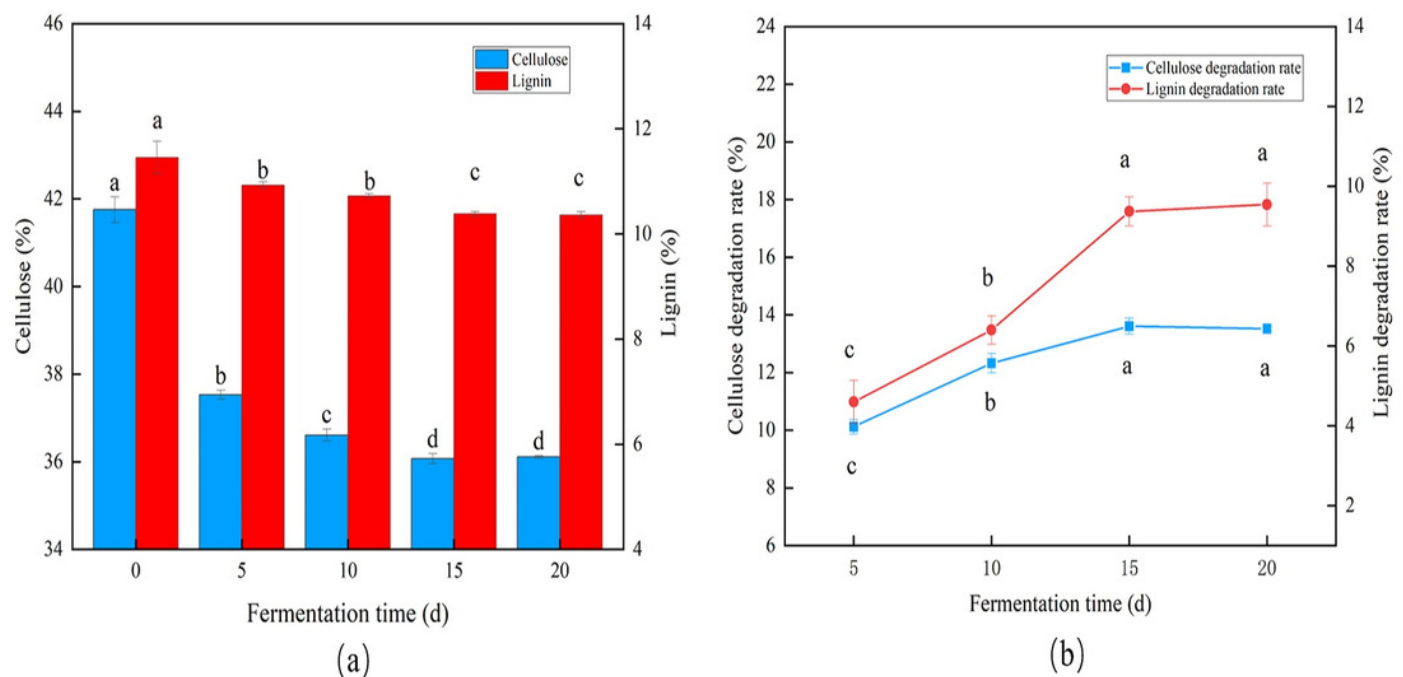
Figure 5

Figure 5 XRD diffraction results of *Codonopsis pilosula* straw
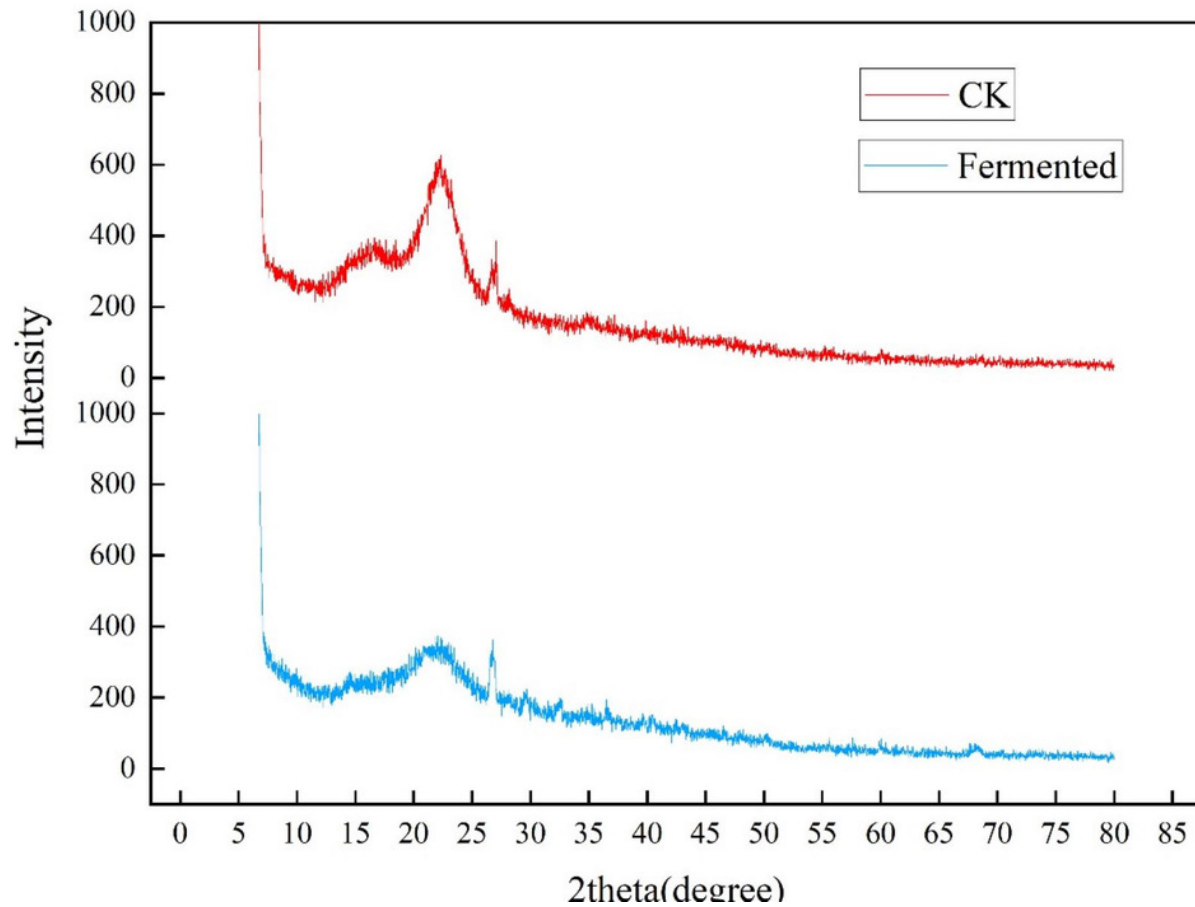
Figure 6

Figure.6 FT-IR results of *Codonopsis pilosula* straw
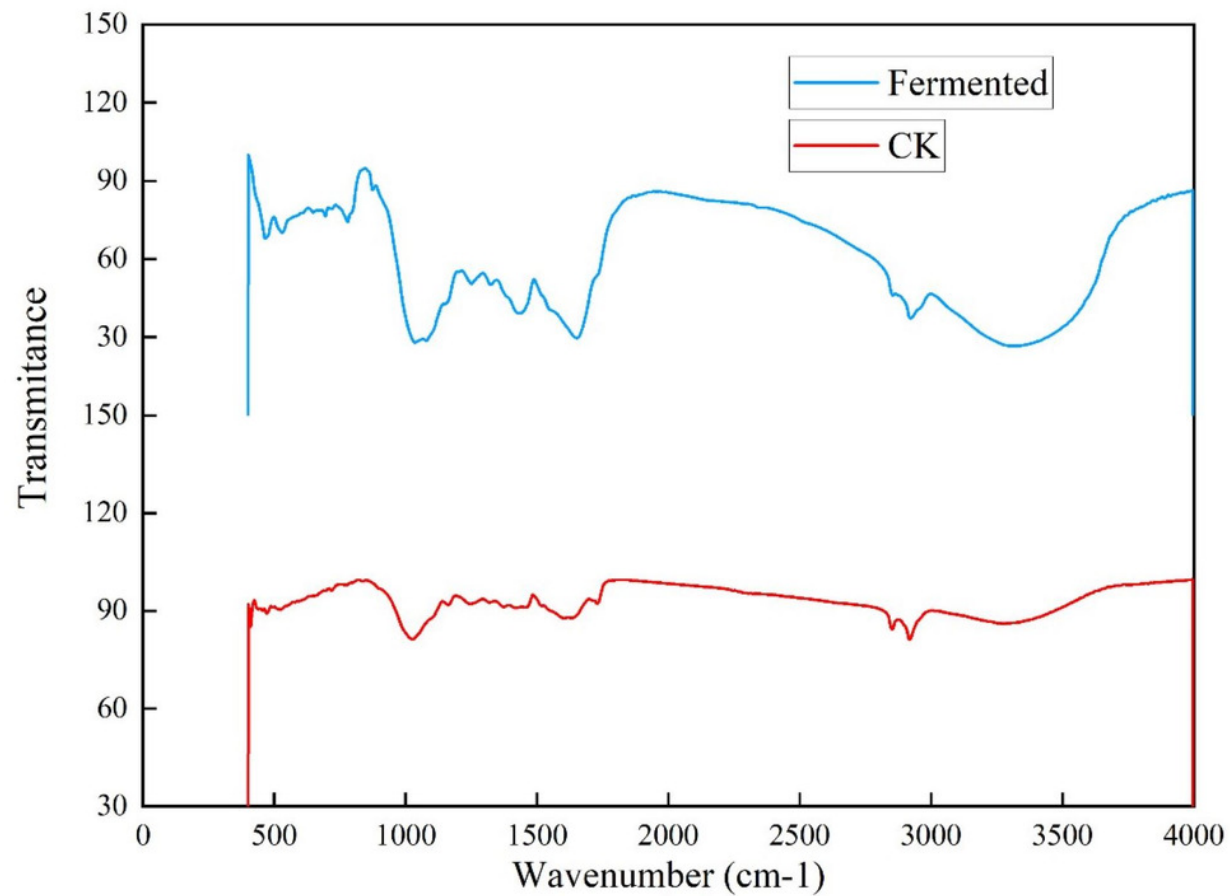
Figure 7

Figure.7 SEM images of Codonopsis pilosula straw

CK   Fermented
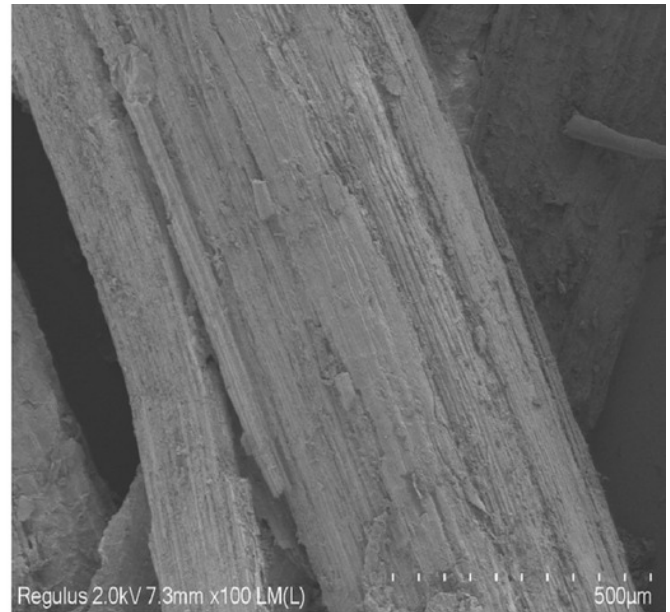
Table 6 The chemical composition of fermented Codonopsis pilosula straw under the optimum conditions

1 **Table 1** Chemical composition (g/kg DM) of *Codonopsis pilosula* straw

Items	Content (g/kg)
Dry matter	905.7±16.32
Crude ash	70.8±2.21
Ether extract	48.4±0.92
Crude protein	154.8±3.55
Acid detergent fiber	541.8±8.24
Neutral detergent fiber	738.7±9.20
Cellulose	417.6±11.36
Lignin	114.6±7.25

Table 2

1   **Table 2** The chemical composition of fermented *Codonopsis pilosula* straw under the optimum
2   conditions

Items[1]	Content (g/kg)
	Fermented
Dry matter	334.7±11.76
Crude ash	72.4±2.21
Ether extract	63.7±1.91
Crude protein	153.6±1.61
Acid detergent fiber	473.3±4.77
Neutral detergent fiber	683.8±5.93
Cellulose	360.6±8.10
Lignin	102.3±3.62