# EFFECTS OF EXTRUSION ON STRUCTURE OF SPENT GRAIN CELLULOSE AND pH VALUE MAINTENANCE DURING SOLID-SUBSTRATE FERMENTATION*

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**Abstract:** The production of low-cost cellulase enzyme is a key step in the development of an enzymatic-based process for conversion of lignocellulosic biomass to ethanol. Although abundant informations are available on cellulase production, little work has been examined on the effects of substrate pretreatment on pH value and cellulase formation during solid-substrate fermentation. This paper investigated pH value changes of substrate during fermentation of *Trichoderma reesei* on native and extruded substrates. After single-screw and twin-screw extrusion, cellulose crystallinity of spent grain was reduced slightly. Single extrusion fragmented and opened cellulose structure, while twin extrusion destroyed and disrupted the cell wall structure of spent grain as well as reduced particle size. Both extrusions favor micro-organism growth and enhance the production of cellulase. Maximal FPA activity of 182.8 IU/g cellulose was observed when spent grain by single extrusion was used as substrate.

**Key words:** solid-substrate fermentation; spent grain; cellulase; *Trichoderma reesei*

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# 挤压对麦糟纤维结构和固态发酵过程中 pH 值影响的研究

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摘 要: 低成本纤维素酶的生产是酶法转化纤维质生物量为酒精的关键。尽管已有很多有关纤维素酶的研究报告,但底物的预处理对固态发酵过程中 pH 值和产酶的影响很少见诸于报道。作者研究了在固态发酵过程中,里氏木霉在未处理和经挤压处理的麦糟培养基上 pH 值的变化。经单螺杆和双螺杆挤压的麦糟,其纤维的结晶度变化较小。单螺杆挤压撕开了纤维结构,而双螺杆破坏并摧毁了纤维细胞的细胞壁,同时麦糟的颗粒也变得很小。两种经挤压处理的麦糟均有利于菌体的生长,并提高了产酶。以单螺杆挤压处理的麦糟为培养基时,最高 FPA 酶活力为 182.8 IU/g 纤维素。

关键词: 固态发酵; 麦糟; 纤维素酶; 里氏木霉

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Solid-substrate fermentation(SSF) is generally defined as that in which microbial growth and product formation take place on a solid substrate in the absence of free water[1]. Several reviews are available in describing the technique[2-4]. In recent years, SSF technology has received increasing interest. This is because it has lower energy requirements and produces less wastewater than submerged fermentation. In addition, there are increasing environmental concerns regarding the disposal of solid wastes, and their use as substrates for the commercial production of microbial metabolites is becoming an attractive proposition.

Because of mass transfer limitations and limited access of microorganism to the substrate, the restriction effect of nutritional factors could be very severe in SSF. During cellulase production, this situation may be more restrict because very few microorganisms or cellulase can break down lignocellulose to be used as substrate efficiently for biomass or other end product formation[5]. For the purpose of developing a practical process of cellulose utilization, enzymatic hydrolysis, which converts cellulosic materials into glucose, together with the pretreatment methods has been extensively studied[6-10]. However, little work has been focus on the effect of cellulose pretreatment on the enzyme production during SSF. In SSF, surface adhesion plays a very important role in fungal growth and mycelial spread. Moreover, enzymatic digestion may occur in advance of hyphae tip, thus facilitating penetrating deep into the interior of the substrate particles[5]. This suggests that pretreatment of substrate may favor the growth of microorganism and formation of enzymes.

On the other hand, pH value is one of the most important environmental factors for cell growth and product formation, but the control of pH value during SSF fermentation was not usually attempted. Kadam *et al*[11] reported the maintenance of pH value around 4.5 is very crucial for cellulase production. Tsao *et al*[12] suggested that optimal pH value for growth and production phase is pH value 4.0 and 3.0 respectively in submerged fermentation. In our previous study, it was found when pH value of substrates increased to 6.0-6.2 during SSF, cellulase activity ceased to increase or even declined. pH value regulation of substrate by spraying inorganic acid could prolong enzyme formation period and therefore enhance cellulase productivity[13]. However, the characteristics of solid substrate limits the uniform distribution of diluted acid and could lead to local excessive acidification.

Objective of this work was to evaluate the effects of extrusion treatment on structure of spent grain cellulose and the pH value maintenance, along with cellulase formation during SSF with *Trichoderma reesei*.

## 1 Materials and methods

### 1.1 Materials

Several substrates were used for SSF as follows: spent grain obtained locally was dried(70 ℃ to constant weight); corncobs were dried, chopped and ground to 40-mesh size; the content of cellulose is 22% and 31.8% respectively. All other chemicals were purchased from Sigma Co. locally.

### 1.2 Extrusion

For each run, spent grain was conditioned to the desired moisture content. Material was placed in sealed polyethylene bags and allowed to equilibrate for 16 h at room temperature, and then was extruded in a single-screw or twin-screw extruder(CREUSOT-LOIRE CLEXTRAL BG-21, France). The extruder was operated at steady state for each set of conditions. Attainment of steady state was judged by constant amperage. Samples were then collected, dried at 80 ℃ to 13% moisture.

### 1.3 Microorganism and media

*T. reesei* Rut G-30 was used throughout this study. The seed liquid medium was composed of(g/L): spend grain 40, glucose 5, pH value 6-6.5. The medium was autoclaved at 121 ℃ for 45 min. The SSF medium was(g/100 g): spent grain(whatever native or extruded) 80, corncobs powder 20.

### 1.4 Cultivation conditions

For inoculants preparation, the microorganism from fresh slant culture was inoculated in 100 mL fresh seed medium in 500 mL flasks and cultured on a rotary shaker at 180 r/min for 4– 5 d at 30 ℃. Inoculants with size 10% of inoculation was transferred to the SSF medium.

For each tray in SSF, 400 g substrate was moistened with 600 mL tap water. pH value was adjusted to desired initial level before autoclaving at 121 ℃ for 40 min. After cooling, the medium was put into a clean porous tray(40 cm × 25 cm × 12 cm). The contents of each tray were mixed thoroughly with a sterile inoculation needle after inoculation. The trays were incubated at 28– 30 ℃ in a humidity-control/maintain room (90% – 95% humidity). The contents of the trays were sampled and assayed periodically for enzyme activity and pH value. Each experiment was done in triplicate and results are mean values of such results.

### 1.5 Analytical methods

Activities of cellulase on filter paper were determined according to Mandel's method[14]. Briefly, crude cellulase was extracted from fermented solids with buffer. Filter paper activity(FPA) was assayed by incubating 0.5 mL of suitably diluted enzyme with Whatman No. 1 filter paper strip(6 cm × 1 cm) and 1.0 mL of buffer solution(pH value 4.8) at 50 ℃ for 60 min. The reaction was terminated by adding 1.5 mL DNS and boiling for 5 min. Reducing sugar was analyzed by DNS method using glucose as standard. One unit (IU) of enzyme activity was expressed as the amount of enzyme that liberates 1 μmol glucose per minute per gram cellulose under assay conditions.

Measurements of pH value of solid substrate were made by extracting a sample of fermented substrate with cool boiled distilled water as described by Morre and Jonson[15]. The percentage moisture in the solid substrate was determined gravimetrically after drying the samples in an oven at 105 ℃ for 24 h.

Scanning electron micrographs(SEM) were obtained using a SX40 microscope with 10 kV acceleration. Samples were dried over silica gel at 35 ℃, during 3 d to eliminate residual moisture, coated with gold in high vacuum and examined in the microscope. X-ray diffractometry of samples was obtained with a D/MAX-RA unit(Ricon, Japan). Settings were 40 kV, 20 mA. The samples were scanned for a range of 2θ from 6° to 46°. $I_{002}$ is the intensity of the 002 peak(at about 2θ= 22°). The $I_{002}$ peak corresponds to the crystalline fraction as described by Fan *et al*[16].

## 2 Results and discussion

### 2.1 pH value profile during SSF

Fig. 1 showed the typical pH value profile of Koji when *T. reesei* was cultivated on medium composed of native spent grain and corncob. The authors insistently observed that pH value dropped to 3.0 after 50 h cultivation and maintained at this point for about 15 h, and then increased back sharply(0.05 point/h). The previous research indicated that pH value dropped because of acid production during the growth of cells[17]. Rapid decline of pH value corresponded to the utilization of solid substrate. This was confirmed by our observation that there is a heat-producing peak around 50 h when the maximum growth of hyphae took place[13]. As the fermentation went ahead, numbers of amine were produced by cells and resulted in increasing back of pH value.

The native spent grain is barley hull whatever after brewery process, which is composed of cellulose, hemicellulose and lignin. Its high crystallinity is the first, fundamental obstacle to its hydrolysis and utilization. For hydrolysis of cellulose, enzyme must bind to the surface of cellulose molecule to catalyze the reaction. Cellulase enzymes have a molecular weight of 30 to 60 ku and an ellipsoidal shape with major and minor dimension roughly 3 nm and 20 nm respectively. Consequently, only 20% of the pore volume is accessible

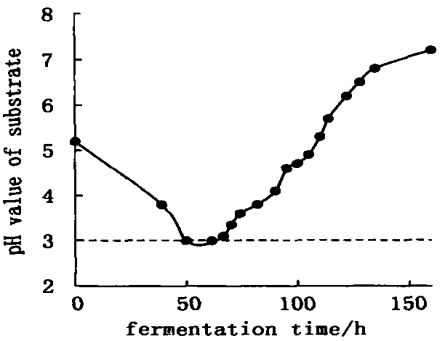
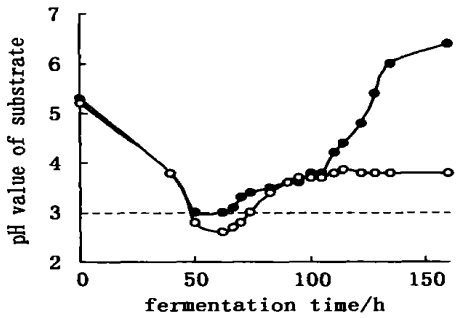



Fig. 1 The typical time course of Koji pH value in trays of native spent grain




—●—single-screw; —○—twin-screw

Fig. 2 Curves of pH value during SSF of extruded spent grain

to cellulase molecules[18]. Obviously, in SSF the utilization of cellulose is very limited because the usable cellulosic substrate is limited. In our previous work, we found that lower pH value could enhance the production of cellulase during SSF[13]. So the next step is to evaluate the effect of extruded material on pH value changes and enzyme production.

## 2.2 pH value profiles during SSF and enzyme production using extruded spent grain as substrate

Lignocellulosic materials are rather resistant to enzymatic hydrolysis unless a suitable pretreatment is used. The intention of the pretreatment is to open the structure of the lignocellulosic material, thereby making it accessible to enzymes. The pH value profiles during SSF using spent grain extruded by single and twin screw were showed in Fig. 2. The lowest pH value of 2.6 was obtained after 50 h cultivation for twin extruded spent grain, compared to pH value 3.0 with single extruded and native spent grain(Fig. 1). Fig. 2 also showed that the maintaining period at low pH value for twin extruded substrate was slightly longer(25 h) compared to single extruded and native spent grain. For twin extrusion the pH value increased steadily, however never exceeded pH value 4.0 during whole fermentation process. At the meantime, two increased slopes profile can be seen for single extrusion. Before 105 h, the pH value increased steadily until pH value 4.0. After that point, it increased more sharply. Table 1 compares the effects of extruded spent grain on the production of cellulase during SSF. For single one, a maximal activity FPA of 182.8 IU/g cellulose was observed after 135 h fermentation, which is 51.1% higher than native spent grain. For twin one, FPA activity of 165.8 IU/g cellulose was obtained.

## 2.3 Comparison of SEM and crystallinity

Fig. 3 compares the microscopic structure of native, single and twin extruded spent grains respectively. Results suggested that there were lots of changes in cellulose structure after extrusion. In Fig. 3(b), the structure was well organized but showed fissures and fragmented structure, which could facilitate water absorption and fiber swelling. This structure, more open than the native material[Fig. 3(a)], could improve fermentable functionality related to these properties. Fig. 3(c) of twin-extruded products showed further disruption of the fiber structure as increased porosity and reduced particle size. During enzymatic hydrolysis process, fragmentation can greatly increase the accessible surface area, depending on the porosity and cellulose particle size[19]. Hydrolysis occurs initially at the external surface of the fibers. In our present study, microscopic examination showed a slight effect on the

Table 1 Effects of extruded spent grain on the production of cellulase during SSF

| spent grain | initial pH value | moisture /% | FPA cellulose/(IU·g$^{-1}$) 114 h | 135 h | 148 h | increase /% |
|---|---|---|---|---|---|---|
| single | 4.6 | 66.42 | 161.9±2.3 | 182.8±2.5 | 180.1±3.0 | 51.1 |
| twin | 4.6 | 65.34 | 110.9±1.8 | 127.1±2.1 | 165.8±2.9 | 37.1 |
| native | 4.7 | 65.65 | 78.5±1.9 | 112.9±1.5 | 120.9±1.8 | – |

hulls epidermis but quite open after single-extrusion, while twin-extrusion promoted cellular structure disruption. Like other hyphae fungus, *T. reesei* grows by apical extension of multinucleate cells called hyphae. Apical cells have active mitotic cycles. It has been reported that the degree of substrate utilization depends upon the capability of fungal mycelia penetrating deep into the intercellular spaces[20]. Obviously, fragmentized and porous substrates were benefit to this hyphae tip growth pattern.


a. native; b. single-screw extruded; c. twin-screw extruded

**Fig. 3　Scanning electron micrographs of spent grain**

Crystalline fractions of native and extruded spent grain, which were measured in this work with an X-ray diffractometer, are shown in Table 2. No significant changes in the X-ray diffraction pattern were observed( data not shown), only the crystallinity $I_{002}$ was reduced slightly compared with spent grain. Fan *et al*[16] found hydrolysis rate is mainly depended upon the fine structural order of cellulose that can be represented by crystallinity rather than the surface area. The correlation relating cellulose porosity and its digestibility is explained as a consequence of the lower crystallinity and easier fragmentation of the more porous celluloses during hydrolysis. When hydrolysis rate is not a problem in SSF, fragmentation of cellulose plays a more crucial role in digestibility and the formation of cellulase.

**Table 2　Crystallinity comparison of native and extruded spent grain determined by X-ray diffractometry**

| spent grain | 2θ/° | crystallinity, $I_{002}$ |
|---|---|---|
| native | 22.076 | 1499 |
| single extrusion | 22.105 | 1433 |
| twin extrusion | 22.120 | 1369 |

## 3　Conclusion

Enzyme production during SSF is greatly influenced by three integrating factors: cultivation system, microorganism and substrate[1]. Cellulases are inducible enzymes. Substrate can induce synthesis of cellulase, but the nature of substrate also influences enzyme production because of limitations of mass transfer and the low efficiency of utilization of cellulose. On the other hand, pH value of substrate can influence the growth and the production of enzyme. We show in this report that single-extruded spent grain favors the formation of cellulase as well as long phase maintenance of pH value during SSF. Cellulose crystallinity appeared to be only slightly affected by pretreatment of extrusion. Most of the modification achieved in the pretreatment was probably the porous and fragile structure formed. Our data underline the role of single-extrusion of spent grain in enhancement of cellulase production.

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