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Depolymerization of Chitosan with a Crude Cellulase Preparation from *Aspergillus Niger*

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Abstract A crude cellulase preparation from *Aspergillus niger* was used to depolymerize chitosan. The depolymerization process was followed by measuring the apparent viscocity and the intrinsic viscosity. The optimum conditions for enzymatic hydrolysis were investigated. On the selected optimum conditions (pH 5.0, temperature 50 °C, and an enzyme to substrate ratio of 1:5), chitosan was hydrolyzed for 1, 4, 8, and 24 h, its viscosity-average molecular weights were 3.49×10^4, 1.18×10^4, 5.83×10^3, and 1.13×10^3, respectively. Compared with chitosan having viscosity-average molecular weight of 5.18×10^5 before enzymatic hydrolysis, the crude cellulase preparation had rather apparent effect on depolymerization of chitosan. Through the comparison of different origin of cellulases, the prepared cellulase has good ability of enzymatic hydrolysis. The reproducibility and reversibility for enzymatic hydrolysis was appraised. The data are of value for the production of low-molecular weight chitosans and chitooligomers of medical and biotechnological interest.

Keywords Chitosan · Depolymerization · Enzymatic hydrolysis · Molecular weight · Viscometric feature

Introduction

Chitosan, a linear polymer composed of β-1,4-linked D-glucosamine residues with various degrees of *N*-acetylated residues, is a deacetylated derivative of chitin extracted from an abundant source of shellfish exoskeletons [1]. The polysaccharide and its derivatives have displayed extensive and unique value of application in chemical industry, environmental protection, functional food, cosmetics and pharmaceutical industries, biological medicine, and biomaterial [2–6].

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However, the molecular weight of chitosan prepared by deacetylation of chitin is very high and it is insoluble in general solvents. It only can be dissolved in some acidic media, which makes very much limit to the application of chitosan. On the other hand, studies have shown that the molecular weight has much effect on the property of chitosan [7, 8]. The properties of chitosan with different molecular weights vary very much. Sometimes it even takes on contrary traits [9]. Many special functions of chitosan are displayed when the molecular weight declines to some extent. So it is very essential to select a proper method of degradation.

At present, the method of degradation of chitosan mainly includes chemical degradation, physical degradation, and biodegradation [10]. The chemical degradation utilized by industrial production comprises acid degradation and oxygenated degradation. It has great difficulty in controlling the conditions of reaction and separation. Moreover, the efficiency of producing polymer with degree of polymerization beyond the six range is low and the cost is high [11, 12]. Studies on the physical degradation are relatively less. Thereinto, ultrasonic degradation is able to get comparatively uniform low-molecular weight chitosan [13]. However, owing to low production efficiency and high production cost, the physical degradation still demands further research. Biodegradation, namely enzymatic degradation, owns many advantages. It has selectivity and can choose to split specific chains, from which chitosans with specific molecular weight can be obtained. In addition, the course of hydrolysis and product distribution can be controlled more readily and the enzymatic method also minimizes alterations in the chemical nature of the reaction product [14, 15]. Therefore, the enzymatic degradation is regarded as the optimal method of degrading chitosan. Of studies on the enzymatic degradation, researches on chitinase and chitosanase are the most extensive [14, 16, 17]. But chitinase and chitosanase is currently difficult to obtain in bulk quantity for commercially viable applications [18].

Cellulase constitutes a complex enzymatic system responsible for the degradation of cellulolytic substances. It occurs widely in various fungi, bacteria, insects, and other lower animals. It has not been mentioned very often in conjunction with chitosan. Recently, an unspecific hydrolytic action of cellulase from *Trichoderma viride* on chitosan at acidic pH was reported [19]. The present paper describes the unspecific hydrolysis of chitosan by a crude cellulase preparation from *Aspergillus niger*, which is an alternative economical way of obtaining low-molecular weight chitosans and chitooligomers.

Materials and Methods

Materials

Chitosan was purchased from Qingdao Haisheng Bioengineering. It originated from crab shell and its degree of deacetylation was 84%. Its apparent viscosity was 333 mPa s (2% of chitosan solution, measured by the NDJ-circumrotate viscometer, Shanghai Precision Scientific Instrument). Its viscosity-average molecular weight was 5.18×10^5 (measured by the 1835-Ubbelohde viscometer, Shanghai Glass Instrument No.1 Factory). Crude cellulase preparation was prepared with liquid-state fermentation by our lab using *A. niger* as enzyme-producing strain. *A. niger* No.5.1 was provided by the Culture Collection and Research Center of Microbiology, Beijing Normal University, China. It was cultivated in the medium containing cellulose powder as the sole carbon source at 32 °C. The medium was prepared by the method of Cappellini and Peterson [20]. An inoculum of spore

suspension of the fungus containing 1.0×10^7 spores was introduced into 50-mL culture solution. After 5 days of growth at 120 rpm, the medium was filtered through sintered glass filter. The filtrate obtained has been designated as "culture filtrate". The crude enzyme preparation was obtained by precipitation using solid ammonium sulfate and centrifugation at 4,000 rpm for 30 min.

Purification of Chitosan

Chitosan was further purified before use. Twenty grams of chitosan was dissolved in 1 L of 1% (*v*/*v*) acetic acid solution. After it was filtered under diminished pressure, 1 M of concentrated alkali (NaOH) was added into the filtrate to produce precipitate. The precipitate was washed repeatedly with deionized water until the rinse water was at a neutral pH. Two hundred milliliters of anhydrous alcohol was then added to remove a little red pigment in the chitosan. Finally, the precipitate was dried in a vacuum dry box under diminished pressure. The purified chitosan was milled to power to be used for the following experiment.

Enzymatic Hydrolysis of Chitosan

Accurately weighed chitosan was dissolved in 1% (*v*/*v*) of acetic acid solution. One percent (*w*/*v*) of enzyme solution was then added to the chitosan solution. The reactions were conducted by incubation at different temperatures in a temperature-controlled water bath shaker. The reaction time was varied to obtain material with different degrees of polymerization. At the end of the specific time period, the reaction was stopped by boiling the mixture for 10 min to denature the enzyme. Then the enzyme was removed by filtration under reduced pressure.

Measurement of the Apparent Viscosity

The viscosities of the chitosan solution before and after reaction were determined by DNJ-circumrotate viscometer at 25 °C.

Measurement of the Intrinsic Viscosity

Chitosan solutions of known concentrations were prepared in a solvent system consisting of 0.5 M acetic acid and 0.25 M sodium chloride in deionized water. The solutions were then filtered through a 0.45-μm millipore filter prior to the viscosity measurements. The viscosity measurements were made, in triplicate, by recording the efflux times of the filtered solutions in Ubbelohde viscometers maintained in a constant-temperature water bath at 25 °C.

Determination of Chitosan Viscosity-Average Molecular Weight

The viscosity-average molecular weights of chitosans were calculated from the classical Mark–Houwink relationship: $[\eta]=K\,(M_w)^{\alpha}$, where $[\eta]$ is the intrinsic viscosity of chitosan/depolymerized chitosan, M_w is the viscosity averaged molecular weight, and K and α are constants that depend on the polydispersity of chitosan and the solvent system used. The values of these constants were previously determined to be $K=1.424\times10^{-3}$ and $\alpha=0.96$ at 25 °C [21].

Results and Discussion

Optimization of Experimental Conditions for Enzymatic Hydrolysis

To optimize depolymerization of chitosan, the effects of the following factors during the depolymerization step were studied: pH (2.0–6.0), temperature (40–60 °C), and enzyme-to-substrate ratio (1:20–1:5, *w*/*w*).

pH Studies

For pH optima determination, chitosan solutions were adjusted to different pH values with HCl and NaOH, followed by incubation with enzyme (100 mg solid/g chitosan) at 50 °C. Half-unit increments were prepared and tested for viscosity reductions at 25 °C as previously stated. Comparison of hydrolytic efficiencies at pH 2.0–6.0 was carried out using chitosan at 1.0%. Aliquots (20 μL) of enzyme (10 mg solid/mL in distilled water) were incubated with mild mixing using an orbital shaker (100 rpm) at 50 °C. Samples were analyzed for the apparent viscosity at selected time intervals (5, 10, 15, 30, and 60 min), and the viscosity decrease percent (VDP) was calculated as $VDP=(V_0-V_t)/V_0$, where V_0 is the initial viscosity and V_t is the viscosity after certain time of incubation with the crude cellulase preparation. The results were displayed in Fig. 1. It suggested that the enzyme preparation displayed no sensitivity to pH environments, being active at the lower and higher pH values, whereas a pH optimum of 5.6–6.0 was reported for cellulase and chitosan as substrate [22].

Temperature Optima

Chitosan solutions were incubated with enzymes (100 mg/g chitosan) at 40–60 °C in 5 °C increments. Sampling was performed at 5, 10, and 15 min. Viscosities were measured and the viscosity decrease percents were determined as previously described. The result suggested that at 50 °C the enzyme preparation caused significant hydrolysis of chitosan with viscosity decrease of over 90% in 15 min, while at 60 °C it resulted in insignificant hydrolysis with viscosity decrease of about 20% in the same time (Fig. 2). The enzyme was

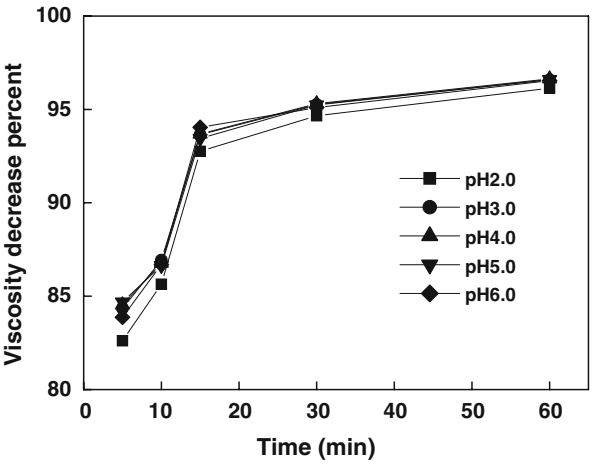

Fig. 1 Effect of different pH on depolymerization of chitosan following treatment with the enzyme preparation at 50 °C and enzyme-to-chitosan ratio of 0.1

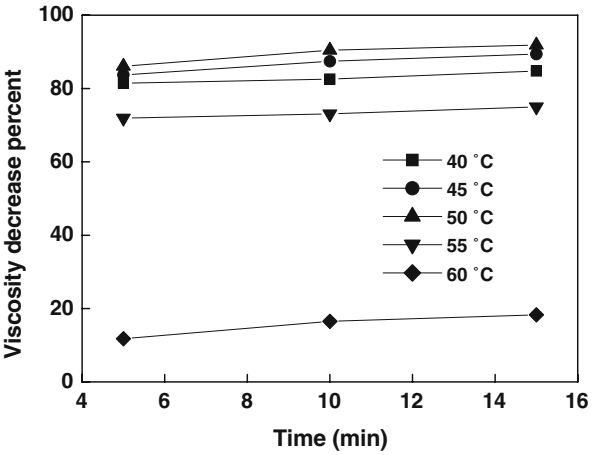

Fig. 2 Viscosity decrease percent for a 1% chitosan solution after enzyme treatment for different times as a function of temperature at pH 5.0 and enzyme-to-chitosan ratio of 0.1

relatively stable in the temperature range below 55 °C, but was rapidly inactivated at higher temperatures.

Dosage Studies

Solutions of chitosan (60 mL) were mixed with different amounts of enzymes in distilled water (2 mL), with enzyme-to-chitosan ratios of 0.05–0.2, and incubated at 50 °C and pH 5.0. Hydrolysis rates were determined by monitoring the reaction viscometrically every certain time intervals and the percent viscosity decrease was plotted vs. the enzyme: chitosan (*w*/*w*) ratio (Fig. 3). The result indicated that a higher enzyme/substrate ratio facilitated the hydrolysis and allowed low-molecular weight chitosan preparation. This is in accordance with studies of other researchers [15, 18].

From the above experiments, some conclusions can be drawn: the crude enzyme preparation had a broad pH optimum. The effect of pH value on enzymatic hydrolysis of

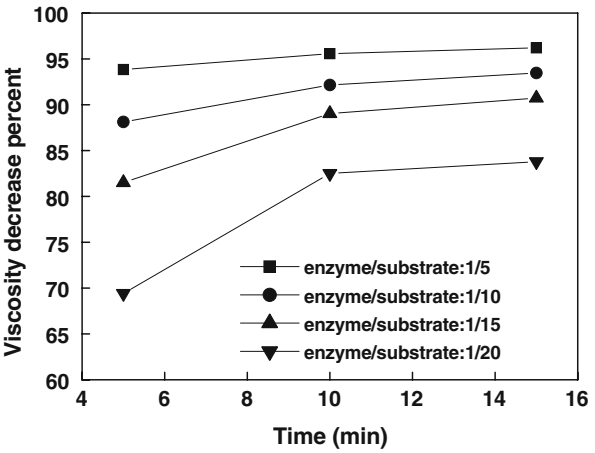

Fig. 3 Dose–response profile of enzymatic hydrolysis of chitosan at different times following treatment with the enzyme preparation at pH 5.0, 50 °C and enzyme-to-chitosan ratios of 0.05–0.2

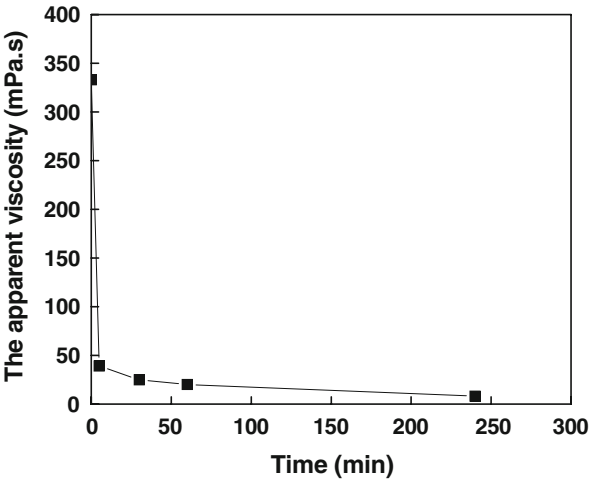

Fig. 4 Viscosities of chitosan solutions as a function of time following treatment with the enzyme preparation at pH 5.0, 50 °C, and enzyme-to-chitosan ratio of 0.1

chitosan was not obvious. In the range of pH 2.0–6.0, the cellulase preparation has good enzymatic activity. The optimum temperature of enzymatic hydrolysis was 50 °C and with the increase of the ratio of enzyme to substrate the velocity of depolymerization speeded up. The following conditions of pH 5.0, 50 °C, and enzyme to substrate ratio of 1:10 were used to investigate the relation of the degradation time and viscosity and count the relative molecular weight.

Depolymerization of Chitosan Under the Optimum Conditions

The results were shown in Figs. 4 and 5. Chitosan was readily depolymerized by the cellulase preparation as shown by the viscosity decrease of its acetate solutions. In the course of the initial 5 min, the consequences of the enzymatic action were quite evident. A

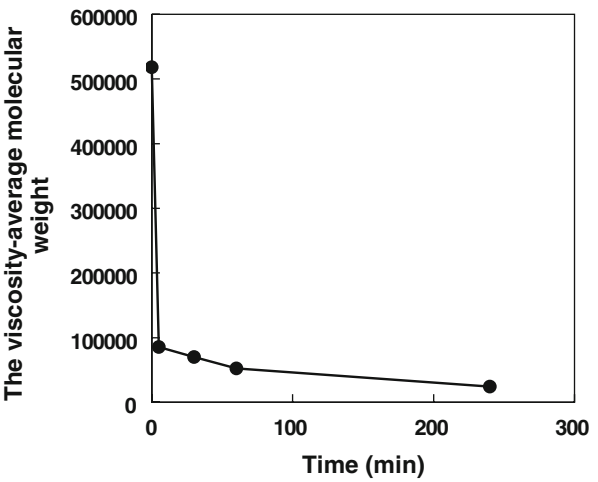

Fig. 5 Effect of enzymolytic time on the viscosity-average molecular weight of chitosan following treatment with the enzyme preparation at pH 5.0, 50 °C, and enzyme-to-chitosan ratio of 0.1

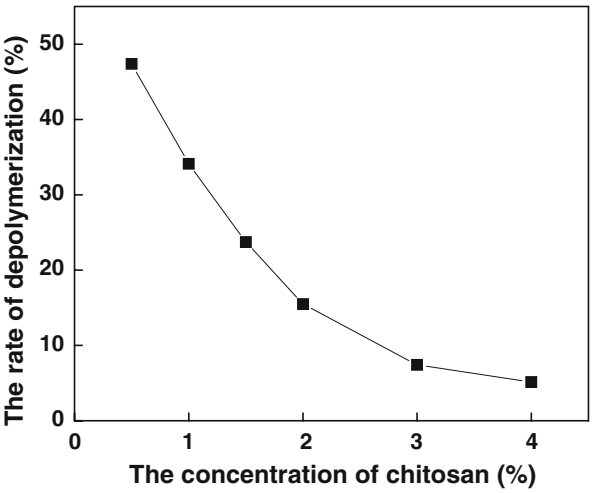

Fig. 6 Influence of chitosan concentration on depolymerization on the conditions of 50 °C, pH 5.0, and enzyme-to-substrate ratio of 0.1

viscosity decrease as high as 89.1% could be obtained in 60 min. On the conditions of pH 5.0, 50 °C, and the ratio of enzyme to substrate 1:10, chitosan was depolymerized for 5, 30, 60, and 240 min, its average molecular weight decreased to 8.58×10^4,7.02×10^4,5.23×10^4, and 2.44×10^4, respectively. In contrast to chitosan with the viscosity-average molecular weight of 5.18×10^5 before enzymatic hydrolysis, the rates of degradation were 16.5%, 13.5%, 10.9%, and 4.7% respectively.

Enzymatic Hydrolysis of Chitosan at Different Concentrations

Chitosan solutions (30 mL) at 0.5%, 1.0%, 1.5%, 2.0%, 3.0%, and 4.0% (*w*/*v*) in 1% (*v*/*v*) of acetic acid solution were treated with the enzyme preparation. Cellulase was added (3 mL), followed by incubation at 50 °C with mild stirring. Samples were removed after 5 min and analyzed for the rate of depolymerization. Figure 6 suggested that reactions at 4% showed insignificant levels of hydrolysis.

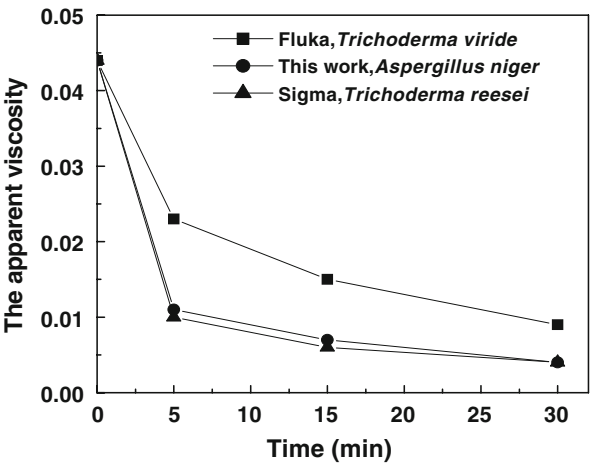

Fig. 7 Comparison of cellulases with different sources on the conditions of 50 °C, pH 5.0, and enzyme-to-substrate ratio of 0.1

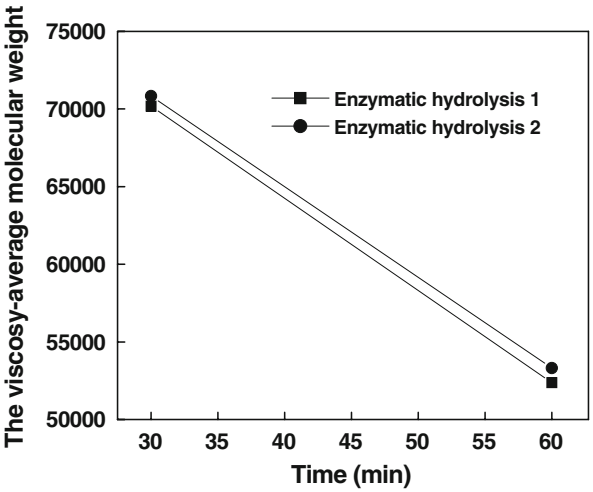

Fig. 8 Reproducibility and reversibility for enzymatic hydrolysis on the conditions of 50 °C, pH 5.0 and enzyme-to-substrate ratio of 0.1

Comparison

Cellulase (from *T. viride*, Fluka), cellulase (from *Trichoderma reesei*, Sigma), and the prepared cellulase (from *A. niger*) were used to depolymerize chitosan on the same condition of 50 °C, pH 5.0, cellulase to chitosan ratio of 1:10. After 5, 15, and 30 min of enzymatic hydrolysis, the apparent viscosity was measured. The results were shown in Fig. 7. It suggested that the effect of cellulase derived from Fluka on depolymerization of chitosan was low, while the effect of cellulase from Sigma and the prepared cellulase on depolymerization of chitosan was high. If the prepared cellulase is further purified, the depolymerization efficiency is obviously higher than that of other cellulases. This is possibly the main reason that cellulases from *Aspergillus* have higher β-glucosidase activity than those from *Trichoderma* [23].

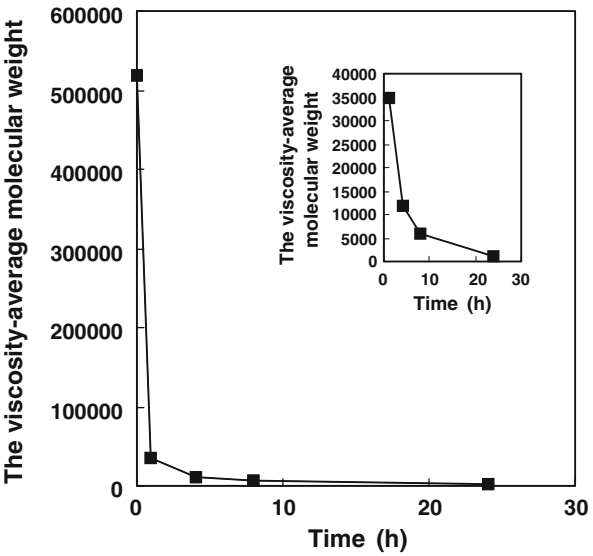

Fig. 9 Time-course of enzymatic hydrolysis of chitosan on the conditions of 50 °C, pH 5.0, and enzyme-to-substrate ratio of 0.2

Appraisement of Precision and Reproducibility

The same with the experiment discussed in Section "Depolymerization of Chitosan Under the Optimum Conditions", under the conditions of 50 °C, pH 5.0, and the ratio of enzyme to chitosan 1:10, the time of enzymatic degradation was 30 min and 60 min, respectively. Check the intrinsic viscosity and calculate the viscosity-average molecular weight. The result indicated (Fig. 8), on the same experimental conditions, the viscosity-average molecular weight of the product by enzymatic degradation of chitosan was stable and the reproducibility was good. This is in accordance with the trait of enzymatic hydrolysis [14, 15].

Long-time Enzymatic Hydrolysis of Chitosan

On the conditions of 50 °C, pH 5.0, and cellulase to chitosan ratio of 1:5, degrade chitosan for 1, 4, 8, and 24 h, respectively and determine the intrinsic viscosities and work out the viscosity-average molecular weights (Fig. 9). The result showed that with the increase of the ratio of cellulase to chitosan and the time of enzymatic hydrolysis, the average molecular weights of chitosan declined to 3.49×10^4, 1.18×10^4, 5.83×10^3, and 1.13×10^3, respectively. The impact of depolymerization was distinctly strengthened. By controlling the reaction conditions, chitosans with different molecular weights can be obtained.

Conclusion

This work indicates that the crude cellulase preparation from *A. niger*, a low-cost commercial enzyme and widely accepted in the food and medicine industries, could be a valid alternative to chitinase and chitosanase, which are expensive and unavailable in bulk quantity for the production of low-molecular weight chitosans and chitooligomers.

Although the usual kinetic models are not applicable in consideration of the unspecific activity of the cellulase preparation, some common conclusions can be drawn in the course of unspecific enzymatic hydrolysis: the viscidity of solution declines quickly in the initial stage of hydrolysis, which suggests that cellulase functions by means of endo-splitting; enhancing the ratio of enzyme to substrate will increase the speed of reaction. It may serve as an effective approach of industrialized production of low-molecular weight chitosans and chitooligosaccharides because the speed of unspecific enzymatic hydrolysis is fast, the condition of production is mild and easy to control, the complicated producing equipment is not demanded, and the cost of production is low.

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