Selective Biodegradation of Grape Pomace Tannins by *Aspergillus niger* and Application in Wood Adhesive

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The selective biodegradation of grape pomace tannins was studied using *Aspergillus niger* strains. The conditions were optimized in terms of monomeric catechin production. The optimal degradation conditions were: 10.0 $g \cdot L^{-1}$ of tannins in the induced medium, pH 6.5, and cultured on a shaking table at a rate of 120 $r \cdot min^{-1}$ at 28 °C for 36 h. The biodegraded tannins were used to partially substitute for phenol-formaldehyde in adhesive formulations. The biodegradation enabled the production of adhesives and wood panels with better properties (lower resin viscosity, higher internal bond strength, and lower thickness swelling). A particleboard in which the resin contained 60% biodegraded grape tannins displayed good performances (IB_{dry} = 0.46 MPa, IB_{wet} = 0.15 MPa) and passed the standard specifications for dry and wet conditions.

Keywords: Grape pomace tannins; Selective biodegradation; Catechin; Aspergillus niger; Phenol-formaldehyde adhesive

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INTRODUCTION

Vegetable tannins are polyphenolic substances that are widely present in the plant kingdom (such as bark, wood, fruits, leaves, roots, and needles) as secondary metabolites for protective purposes, with molecular weights between 500 Da and 30,000 Da. Vegetable tannins are important renewable natural resources and have been used industrially in a wide range of applications. On the basis of their chemical structural characteristics, tannins are divided into hydrolysable tannins and condensed tannins (Venter *et al*. 2012; Radebe *et al*. 2013; Ricci *et al*. 2015). Condensed tannins obtained from natural resources have been used for the production of wood adhesives since the 1970s. They can be used to prepare tannin-based adhesives and/or to replace part of the phenol in phenol-formaldehyde adhesives (Pizzi 1994, 2006; Lei *et al*. 2008; Tondi and Pizzi 2009; Kemppainen *et al*. 2014; Grasel *et al*. 2016).

Grape pomace, consisting of skins, seeds, and stems, is a co-product generated by grape juice and wine-making processes and retains a high level of condensed tannins (20% to 30% of the initial weight) (Llobera and Canellas 2007; Zocca *et al*. 2007; Rondeau *et al*. 2013; Bosso *et al*. 2016). In previous works, the authors described the optimization of the extraction of crude condensed tannin extracts from grape pomace using water as a solvent, in the presence of a base (carbonate or sodium hydroxide) and sodium sulfite. The tannins extracted from grape pomace mainly consist of phloroglucinol A-ring procyanidin polymers with a minor amount of prodelphinidin linked through a C4-C8 linear bond, and a part of procyanidins that contain glucose and gallic acid substituents. It has been demonstrated that these extracts can be successfully employed in the formulation of tannin-

formaldehyde-based adhesives usable for the production of wood panels (Ping *et al*. 2011a,b, 2012). However, it was concluded that, compared with tannins extracted from barks, the performance of some of the pomace-based formulations was poor. This was probably due to the large variability of the extracts and the presence of high molecular weight polymerized procyanidins.

Tannins, especially condensed tannins, are highly resistant to biodegradation because they generally have toxic effects on microorganisms. However, some kinds of aerobic bacteria and fungi (such as *Aspergillus* and *Penicillium*) that can utilize condensed tannins as a carbon source and secrete extracellular enzymes, have been described to have the ability to degrade condensed tannins through the action of tannase enzymes. The tannins were probably first degraded to flavanol oligomers and monomers, and then further degraded into small molecules *via* a ring opening reaction (Grant 1976; Bhat *et al.* 1998; Huang *et al.* 2002; Luo 2006; Hu *et al.* 2015). Nguz *et al.* (1994) reported the degradation of sorghum condensed tannins by *Penicillium*. Gamble *et al.* (1996) used the carbon-13 nuclear magnetic resonance (^{13}C-NMR) technique to compare the tannins' biodegradation ability of two white rot fungi strains in *Sericea lespedeza*. Contreras-Domínguez *et al.* (2006) studied the degradation of procyanidins by *Aspergillus fumigatus* and proposed reaction schemes for the interpretation of the results.

In this work, *Aspergillus niger* was selected for the depolymerisation of grape pomace tannin extracts. The effect of carbon sources, nitrogen sources, incubation time, incubation temperature, pH, and the concentration of grape pomace tannin extracts on the production of monomeric catechin were examined. Finally, the authors verified for the first time the possible use of biodegraded condensed tannin extracts as a component of adhesive formulations, and biodegraded extracts from grape pomace were used as a substitute of phenol in phenol-formaldehyde adhesives. The effect of the selective biodegradation of tannins on the phenolic adhesive properties was also examined.

EXPERIMENTAL

Materials

Commercial poplar veneers (*Populus euramericana*) were purchased from Anhui Tiankang Timber Co., Ltd., Tianchang, China. Grape pomace was provided by Hebei Changli Winery, Qinghuangdao, China. The paraffin emulsion was purchased from Linyi Huacheng Chemicals Co., Ltd., Linyi, China. The silica G60 plate was purchased from Merck Chemicals, Shanghai, China. The chromatography paper was purchased from Whatman, Shanghai, China. The chemical reagents used in the Czapek's medium, catechin, and vanillin, were purchased from Sigma-Aldrich Chemicals, Shanghai, China. The other chemicals were purchased from Nanjing Chemical Reagent Co., Ltd., Nanjing, China.

The *Aspergillus niger* strain was obtained by mutagenizing and screening of original *A. niger*, which was obtained from the China General Microbiological Culture Collection center (Beijing, China). The strain was preserved on a sucrose agar slant at 4 °C.

Composition of the Czapek's medium: sodium nitrate ($NaNO_3$) 3.0 g·L^{-1}, magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$) 0.5 g·L^{-1}, potassium chloride (KCl) 0.5 g·L^{-1}, ferrous sulfate heptahydrate ($FeSO_4 \cdot 4\ H_2O$) 0.01 g·L^{-1}, dipotassium hydrogenphosphate (K_2HPO_4) 1.0 g·L^{-1}, sucrose 30.0 g·L^{-1}, and the media were autoclaved for sterilization at 121 °C for approximately 20 min. Composition of the induced culture

media: grape pomace tannin extracts were added to the Czapek's medium, and the culture media were autoclaved at 121°C for 20 min.

Methods

Extraction and purification of tannin extracts

Grape pomace tannins were extracted with an aqueous solution of sodium carbonate and sodium sulfite (Ping *et al.* 2011a, 2011b). Acetone was used for the further purification of the tannin extracts to remove the non-tannin substance. An amount of 100 g of the tannin extracts was dissolved in 400 mL of 70% (v/v) acetone solution, and then was ultrasonically extracted at 25 °C for 60 min. The upper supernatant liquid was collected after centrifugation for 15 min. The residues were extracted again with acetone solution. The upper supernatant liquid was combined, centrifuged, and evaporated to a moderate concentration, then dried to yield tannin powder.

Biodegradation of tannin extracts

The effects of different carbon sources on the growth of *A. niger* strains were studied. First, 30.0 $g \cdot L^{-1}$ of glucose, lactose, and sucrose were added as the carbon source. The induced medium was cultured in a shaking table at a rate of 120 $r \cdot min^{-1}$ at 28 °C, in which the concentration of the tannin extract was 10.0 $g \cdot L^{-1}$ and the pH was 6.5. The biomass of the strain was determined by the wet weight of the strain every 6 h.

The effects of incubation time, temperature, pH, and the concentration of the tannin extract on the biodegradation of grape pomace tannins were also studied. The incubation time was studied every 6 h. The temperatures studied were 20 °C, 25 °C, 28 °C, 30 °C, and 32 °C, respectively. The pH was studied at 5.5, 6.0, 6.5, 7.0, and 7.5, respectively. The concentration of tannins studied were 2.5 $g \cdot L^{-1}$, 5.0 $g \cdot L^{-1}$, 7.5 $g \cdot L^{-1}$, 10 $g \cdot L^{-1}$, 12.5 $g \cdot L^{-1}$, and 15 $g \cdot L^{-1}$, respectively.

Determination of condensed tannin and catechin

The contents of condensed tannin and catechin were tested by thin layer chromatography (TLC) and ultraviolet spectrophotometry (UV) according to the method of Yao *et al.* (2002) and Luo (2006). The tannin extracts solution was treated by means of colour-reaction with vanillin-hydrochloric acid reagent at 50 °C for 20 min. Then, the UV absorbance of the condensed tannin was measured at 500 nm by a UV spectrophotometer (Beijing Persee Corp., Beijing, China). The catechin was separated by TLC with standard chromatographic paper in a silica G60 plate using BAW (butanol:acetic acid:water, 4:1:5, *v*/*v*/*v*) as the developing solvent. Next, the catechin was tested by the same UV method as the condensed tannin.

The UV specification curve was prepared using a catechin standard sample. The mass content of the condensed tannin and catechin in the grape pomace tannin extract was calculated according to Eq. 1. The mass concentration of catechin in the culture liquid was calculated according to Eq. 2,

$$\text{Mass content } (\%) = \frac{C_2 \times V_2 \times V_0/V_1}{M_0} \times 100 \quad (1)$$

where C_2 is the concentration of the eluted solution after colour reaction ($mg \cdot mL^{-1}$), V_2 is the volume of the eluted solution after colour reaction (mL), V_0 is the volume of the tannin extract solution (mL), V_1 is the volume of spotted sample (mL), and M_0 is the mass of grape pomace tannin extract (mg).

$$\text{Mass concentration } (\text{mg} \cdot \text{mL}^{-1}) = \frac{C_2 \times V_2 \times V_0/V_1 - M_0}{V_0} \quad (2)$$

In Eq. 2, C_2 is the concentration of catechin in the eluted solution after colour reaction (mg·mL^{-1}), V_2 is the volume of the eluted solution after colour reaction (mL), V_0 is the volume of culture liquid (mL), V_1 is volume of spotted sample (mL), and M_0 is the mass of catechin in the blank control culture liquid without inoculating strain (mg).

Preparation of tannin modified phenol-formaldehyde (PF) adhesive

The phenol-formaldehyde adhesive formulation (mass ratio) was defined based on Gu (1999) as follows: phenol:formaldehyde (37%) = 1:1.3, phenol:sodium hydroxide (40%) = 1:0.32, and phenol:H_2O = 1:1.6, and tannin extracts, biodegraded tannin extracts of grape pomace were used instead of phenol (20%, 40%, or 60%). The specific synthesis process was as follows: 1) The melted phenol, sodium hydroxide, and water were added into reactor that was connected to the water condenser and blender, stirred, and kept at approximately 40 °C to 45 °C. Approximately 80% of the total mass of formaldehyde was slowly added into the reactor. The medium was heated up to 92 °C ± 2 °C for 30 min; 2) The temperature was cooled down to 50 °C-60 °C, and pomace tannins were added and stirred until all of the tannins dissolved. Then, the reactor was heated up to 85 °C to 90 °C for 2 h. The remaining formaldehyde was added into the reactor to continue the reaction; 3) The viscosity of the liquid was tested to monitor the reaction. When the viscosity reached 600-1000 mPa·s^{-1}, the temperature was cooled down to below 40 °C to stop the reaction. The properties of the adhesive were determined according to the China national standard GB/T 14074 (2006). Thermal mechanical study of the adhesives were studied using thermal mechanical analyser (Netzsch TMA). The experiments were conducted by the three-points bending mode at a heat rate of 10°C/ min from 25 °C to 250 °C.

Particleboard manufacture and testing

Single layer laboratory particleboards with dimensions of 400 mm × 400 mm × 14 mm were prepared using a hot press (XLB, Qingdao Yadong Rubber Machinery Co., Ltd., Qingdao, China) at 3.5 MPa maximum pressure, 195 °C press temperature, and 7.5 min press time. Poplar particles were prepared from poplar veneers by crushing and screening. Then, 12 % (w/w based on dry particles) of adhesive resin solid was loaded. Next, 1.5 % (w/w based on dry particles) of paraffin emulsion was used as the waterproof agent. The mechanical properties of the particleboards were tested according to relevant China National Standards GB/T 4897.5 (2003) and GB/T 17657 (2013).

RESULTS AND DISCUSSION

Content of Condensed Tannin and Catechin

Tannin extracts obtained from grape pomace are a mixture of large amounts of dissolved non-tannin substances during the extraction process. Tannin extracts mainly include phenols, sugars and carbohydrates, organic acids, inorganic salts, nitrogenous substances, and pigments (Bianchi *et al.* 2015; Kalyanaraman *et al.* 2015). Acetone was used to further purify the tannin extracts to improve the tannin content. The ultraviolet (UV) quantification of tannins and catechin was performed using a vanillin-hydrochloric acid reagent after a TLC separation. The mass content of catechin and condensed tannin in the crude grape pomace tannin extracts were 0.3% and 56.8%, respectively.

Optimization of Biodegradation Parameters

The biodegradation of the grape pomace tannin extracts by *A. niger* strains was optimized. The induced culture media with different carbon sources were incubated in the shaker. The biomass growth was determined under different incubation times. The effects of the carbon and nitrogen sources, as well as the incubation duration, on the biomass growth were studied. The results indicated that the highest biomass density was reached at 12 h. It is apparent from Fig. 1 that there was growth inhibition when grape pomace extracts were used as the only carbon source. The ability of transformation and utilization of condensed tannins as carbon sources directly by *A. niger* strains seemed weak. The biomass of strains increased noticeably when external carbon sources were added (glucose, lactose, or sucrose) with sodium nitrate as the nitrogen source. The wet weight of strains increased 2.57g in glucose source, 2.07g in lactose source, and 3.31g in sucrose source. The highest wet weight of strains was obtained when sucrose was used as external carbon sources.

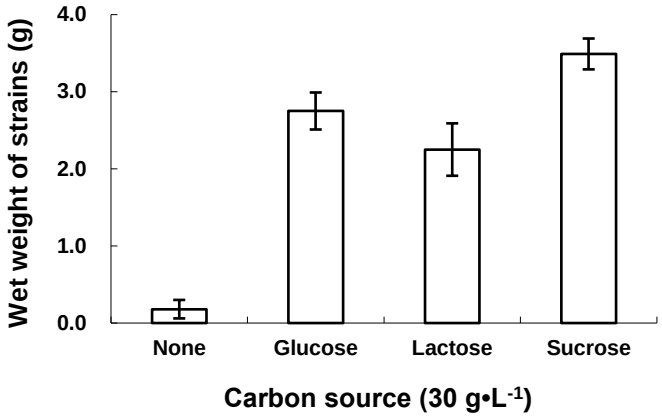

Fig.1. Effect of carbon source on the growth of *A. niger* strains

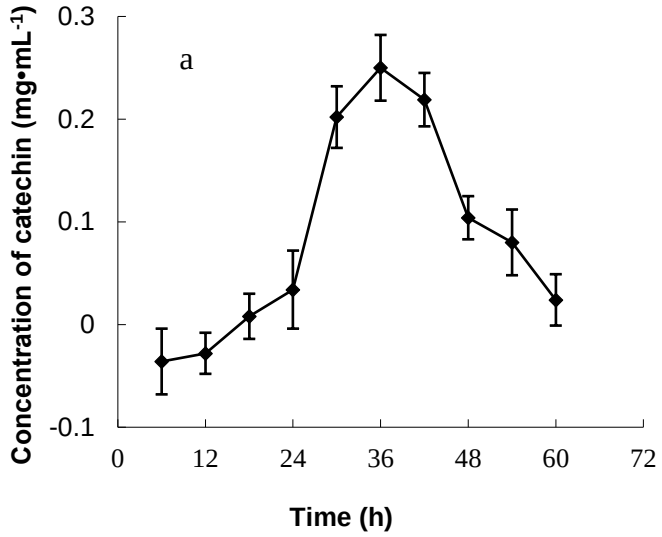

Fig. 2(a). The concentration of catechin in the culture medium as a function of the incubation time (a), temperature (b), pH (c), and concentration of tannin (d)

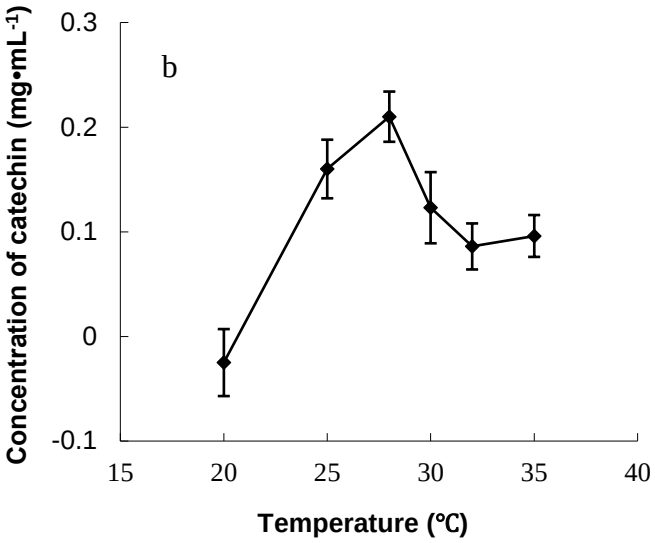

Fig. 2(b). The concentration of catechin in the culture medium as a function of the incubation time (a), temperature (b), pH (c), and concentration of tannin (d)

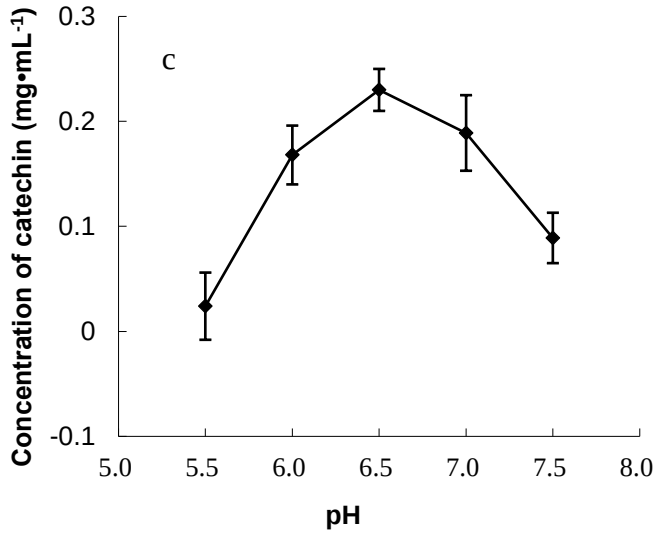

Fig. 2(c). The concentration of catechin in the culture medium as a function of the incubation time (a), temperature (b), pH (c), and concentration of tannin (d)

Based on previous studies, the duration of the incubation is an important parameter for the degradation of condensed tannin extracts into different intermediate products. After a short duration time, flavanol polymers and oligomers are mostly produced. Conversely, extended biodegradation of flavanol monomers into small molecules is expected for a long incubation time (Huang *et al.* 2002; Luo 2006). The concentration of catechin in the culture medium as a function of the incubation time is given in Fig. 2a. The mass concentration of catechin increased to reach a maximum of 0.25 mg·mL^{-1} at approximately 36 h. A further increase of the incubation time led to a strong decrease of catechin due to the reduction of tannins and further degradation of catechin into small molecules.

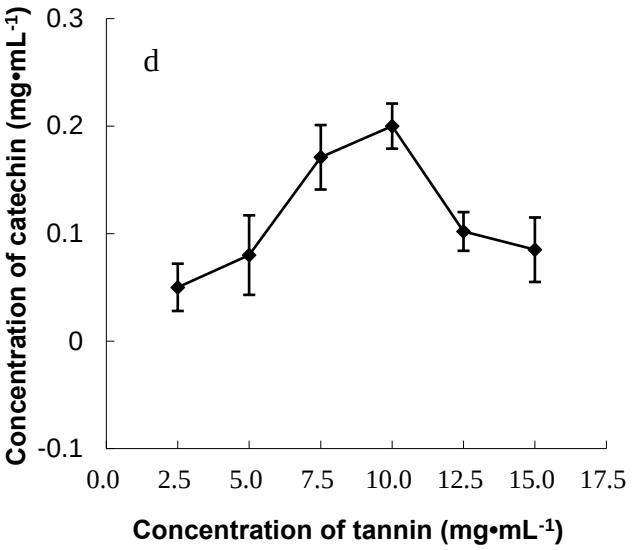

Fig. 2(d). The concentration of catechin in the culture medium as a function of the incubation time (a), temperature (b), pH (c), and concentration of tannin (d)

The other incubation parameters (temperature, pH, and tannins concentration) were also optimized (Fig.2b, 2c, and 2d). It was shown that the optimum catechin production was obtained at pH 6.5, 25 °C to 28 °C, and with a pomace extract concentration of approximately 10 $g \cdot L^{-1}$. It is worth noting that these biodegradation parameters have been optimized in the context of a lab-scale exploratory study. Further improvements should be provided for large scale developments (*e.g.* tannins concentration).

Properties of Tannin-phenol-formaldehyde (TPF) Adhesives

It has been previously shown that the condensed tannins extracted from grape pomace are phloroglucinol-type tannins that are much more reactive toward cross-linking reactions than the resorcinol-type tannins and the phenol itself. In presence of formaldehyde and under acid or alkali catalysis, tannins A-rings can react with formaldehyde to produce polymerization through methylene bridge linkages. As a result, grape pomace condensed tannins can effectively replace phenol in phenolic adhesive production. In this study, crude tannin extracts and biodegraded tannin extracts were used to partially substitute phenol in adhesive formulations. Because of the higher chemical reactivity of tannins, a two steps curing process was developed for the production of TPF adhesives. A pre-reaction of phenol with formaldehyde was first performed to produce low molecular pre-phenolic resin before the addition of tannin.

Phenolic resins containing 20% and 40% of crude grape pomace extracts (F1 and F2, respectively), 20%, 40%, and 60% of biodegraded pomace extracts (F3, F4, and F5, respectively) were examined. The properties of these adhesives are shown in Table 1. In all of the formulations, the free formaldehyde (< 0.3%) and free phenol (< 2.4%) content were low. Compared to PF resin (F0), the free formaldehyde and free phenol contents decreased with the increase of tannin content in the adhesive formulations. It was observed that the pH and viscosity of the resins increased with the pomace extract content. Interestingly, the formulations including biodegraded tannins, F3 and F4, exhibited lower

viscosity than the formulations including crude extracts (F1 and F2). A formulation containing 60% of crude tannins was also prepared, but its viscosity was too high to be further studied.

Table 1. Properties of the Tannin-phenol-formaldehyde (TPF) Adhesives

Formulation Number	Tannin content (%)	Properties of TPF Adhesives					
		Density (kg/m^3)	Solid Content (%)	pH	Viscosity (mPa·s)	Free Phenol (%)	Free Formaldehyde (%)
F0	0	1160	45.4	10.1	620	2.4	0.30
F1	20[a]	1190	46.8	10.2	730	1.6	0.26
F2	40[a]	1210	49.6	11.3	870	1.1	0.19
F3	20[b]	1180	45.5	10.5	650	1.7	0.23
F4	40[b]	1200	48.3	11.6	780	1.0	0.27
F5	60[b]	1230	50.4	12.0	970	0.8	0.21

[a]crude tannins; [b] biodegraded tannins by *Aspergillus niger* strains

The TPF formulations were scanned by thermomechanical analysis in three points bending mode. The MOE curves as a function of the increasing temperature are given in Figs. 3 and 4. The effect of different tannins contents on adhesives MOE values was examined.

It can be seen from Fig. 3 that the adhesive formulations F1 and F2 including, respectively, 20% and 40% of crude tannins gave lower performance ($MOE_{max} \approx 4000$ MPa) than the phenolic resin F0 ($MOE_{max} \approx 5000$ MPa). However, it clearly appeared from Fig. 4 that the utilization of 20% of biodegraded tannins (F3) yielded noticeable improvements in the value of MOE compared to F1, which includes non modified tannins. For higher modified tannins concentrations (40% and 60%, F4 and F5, respectively) a decrease of performance was observed.

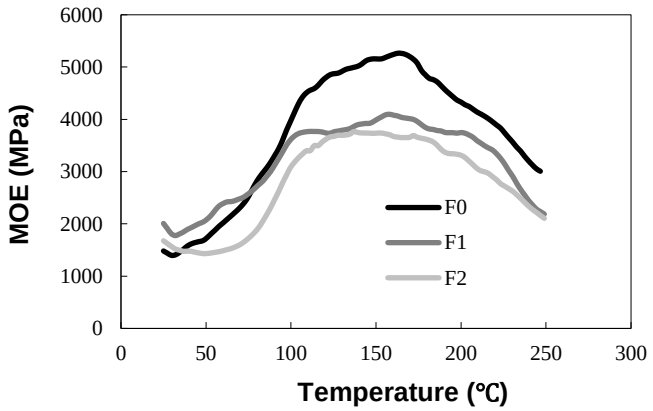

Fig. 3. TMA measuring MOE as a function of temperature to describe the curing of adhesives modified by crude tannins

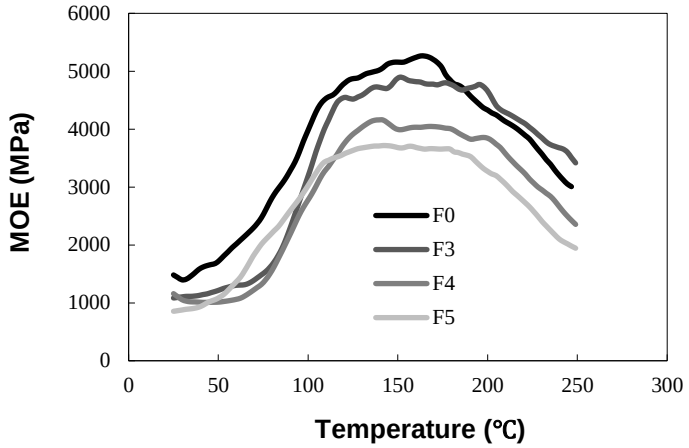

Fig. 4. TMA measuring MOE as a function of temperature to describe the curing of adhesives modified by biodegraded tannins

Properties of Particleboards

The phenolic adhesives (F0 to F5) were used to press one-layer poplar particleboards. The mechanical properties of the particleboards are given in Table 2. All of the panels (F0 to F5) yielded good dry strength (modulus of rupture (MOR), modulus of elasticity (MOE), and internal bond (IB)) that passed the relevant China national standard specification GB/T 4897.5 (2003) for exterior-grade panels (MOR ≥ 16, MOE ≥ 2400, and IB ≥ 0.45).

The IB values are the direct measure of the performance of the adhesive. The results show that the mechanical properties of particleboards decreased with the increase of tannin substitution in adhesive formulations. Interestingly, the particleboards using biodegraded tannins (F3 to F5) were generally superior to the panels produced from F1 and F2 using crude tannins. Especially panel F4 produced from biodegraded tannins, yielded a significantly higher IB strength compared to F2 at the same tannins contents (0.68 MPA and 0.46 MPa respectively).

The particleboards were also tested in wet conditions after cooking in boiling water for 2 h and 24 h for internal bond strength (IB) and thickness swelling (TS), respectively. The results clearly showed that the adhesives including modified tannins (F3 to F5) yielded better wet strength (wet IB and TS) and still met the standard requirements. The formulation F2 including 40% of crude tannins exhibited poor wet results with a low IB and a high TS, and it did not reach the standard requirements. However, the formulation F5, in which the total content of grape tannins was 60% of the total resin, displayed good performances and passed the standard specifications in dry and wet conditions.

The low performance of the adhesive produced from crude tannins is probably due to the high molecular weight and the relatively low chemical reactivity of grape pomace tannins, as previously published (Ping *et al.* 2011b). On the other hand, the biodegradation treatment using *Aspergillus niger* strains produced lower molecular mass tannins with a higher chemical reactivity toward cross linking reactions. As a result, after tannins biodegradation, adhesive formulations and particleboards with improved performance were produced.

Table 2. Mechanical Properties of the Particleboards

Formulation Number	Tannin Substitute Ratio (%)	Dry Conditions				Wet Conditions	
		Density (kg/m^3)	MOR[a] (MPa)	MOE[b] (MPa)	IB[c] (MPa)	IB[d] (MPa)	TS[e] (%)
F0	0	766.7	43.6	3782	0.67 ± 0.03	0.29 ± 0.03	6.2
F1	20	761.5	34.2	3050	0.56 ± 0.05	0.20 ± 0.06	8.7
F2	40	746.3	26.9	2584	0.46 ± 0.02	0.08 ± 0.04	12.4
F3	20	750.7	31.4	2854	0.52 ± 0.02	0.22 ± 0.04	6.9
F4	40	756.4	35.8	3220	0.68 ± 0.04	0.24 ± 0.05	8.6
F5	60	748.2	29.2	2615	0.49 ± 0.01	0.15 ± 0.02	9.4
GB/T 4897.5 (2003)		-	≥ 16	≥ 2400	≥ 0.45	≥ 0.14	≤ 10

The value of each particleboard is the average of three replications; tannins used in formulations F1 and F2 were crude tannins; tannins used in formulations F3, F4, and F5 were biodegraded by *Aspergillus niger* strains; [a] modulus of rupture; [b] modulus of elasticity; [c] dry internal bond; [d] wet internal bond tested after cooking in boiling water for 2 h; [e] thickness swelling tested after cooking in boiling water for 24 h

CONCLUSIONS

1. Grape pomace tannin extracts were biodegraded using *Aspergillus niger* strains. Crude and biodegraded tannins were used to partially substitute for phenol in phenol-formaldehyde adhesive formulations for wood adhesives. Values of free formaldehyde and free phenol decreased, the pH and viscosity increased, and the thermomechanical performance decreased with an increase in the tannins content in the phenolic formulation.
2. The biodegraded tannins allowed the production of adhesives with improved properties in terms of internal bond (IB) and wet behavior.
3. The particleboards bonded by these biodegraded pomace extracts displayed good mechanical properties and passed relevant standard specifications for exterior-grade panels.

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