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A New Detergentless Micro-Emulsion System Using Urushiol as an Enzyme Reaction System

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Abstract Urushiol, a natural monomeric oil, was used to prepare a detergentless micro-emulsion with water and 2-propanol. The formation of micro-emulsion was verified by conductivity measurements and dynamic light scattering. The conductivity data showed phase change dynamics, a characteristic of micro-emulsions, and subsequent dynamic light scattering study further confirmed the phenomenon. Average water droplet diameter was 10 nm to 500 nm when the molar ratio of 2-propanol ranged from 0.40 to 0.44. Earlier studies were performed on toluene and hexane, in which the insoluble substrate in water phase was added to the solvents to be reacted on by enzymes. However, in the present urushiol system, urushiol was used as both solvent and substrate in the laccase polymerization of urushiol. The laccase activity in the system was examined using syringaldezine as a substrate, and the activity increased rapidly near the molar ratio of 2-propanol at 0.4, where micro-emulsion started. The activity rose until 0.46 and fell dramatically thereafter. The study of laccase activity in differing mole fractions of 2-propanol showed the existence of an 'optimal zone,' where the activity of laccase was significantly higher. In order to analyze urushiol polymerization by laccase, a bubble column reactor using a detergentless micro-emulsion system was constructed. Comparative study using other organic solvents systems were conducted and the 2-propanol system was shown to yield the highest polymerization level. The study of laccase activity at a differing mole fraction of 2-propanol showed the existence of an 'optimal zone' where the activity was significantly higher. Also, 3,000 cP viscosity was achieved in actual urushi processing, using only 1/100 level of laccase present in urushi.

Key words: Detergentless micro-emulsion, urushiol, polymerization, laccase, 2-propanol, *Rhus vernicifera*

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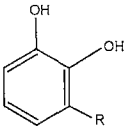

	R	%
Triene	8'c, 11' t, 13'c	67
Diene	8'c, 11't	2.5
	8'c, 11'c	1.2
Monoene	8'c	20.6

Fig. 1. The major congener constituents of urushiol.

Urushiol is an alkenylphenol that constitutes a major portion of the oily constituent of urushi sap from *Rhus vernicifera* (Fig. 1). It contains a catecholic head group with a C-15 tail with varying degrees of saturation [6, 22]. The sap has been used to make Japanese lacquer, which has a beautiful surface quality as well as unsurpassed durability, and the structural integrity is such that thousands of years-old artifacts still retain a shine as new as today [4, 15]. It also has industrially applicable qualities such as hardness, flexibility, chemical inertness, anticorrosiveness, low surface tension, electrical insulation, and antifouling. However, the use has been limited, due to difficulties involved in application and handling of the material. The urushi sap contains 60–70% urushiol, 20–30% water, 10–15% gum and polysaccharides, and less than 1% of enzymes and proteins [2, 5, 21]. The polymerization is driven by laccase, a copper containing oxidoreductase that uses oxygen [1, 18, 19]. The most immediate and central problems are the low laccase activity and the industrial standardization of pretreated urushi: Urushi sap is a natural product, and the collection method and time of collection can result in a great variance in enzyme activity and composition ratio. Development of enzymatic polymerization of purified urushiol and controlled reconstitution of the components can be a viable solution. In this study, urushiol has been used to prepare a detergentless micro-emulsion (DMS) as a possible system for enzymatic polymerization of urushiol.

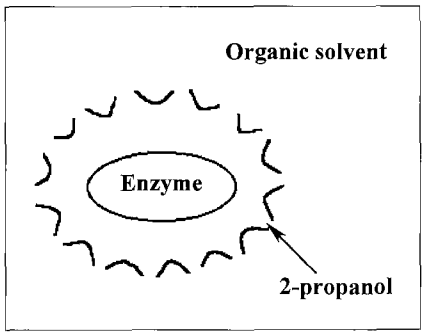

Fig. 2. Possible mechanism of 2-propanol in formation of detergentless micro-emulsion.

Martineck *et al.* [17] proposed DMS as the enzyme reaction system for water insoluble substrates. The formation of micro-droplets of water in hydrophobic organic solvents using 2-propanol was unique, in that thermodynamically stable micro-emulsion was formed without use of any surfactants. It was proposed that 2-propanol was able to carry out the surfactant-like function to create a stable phase separation between water and hydrophobic organic solvent media (Fig. 2). All other water in oil micro-emulsion systems use surfactants to create reverse micelles. There are also studies on enzymatic and cellular reaction optimizations but they are emulsifying systems [10, 11]. Micro-emulsion systems provide an aqueous environment for the enzyme, and the reaction surface area can theoretically increase up to 100 m^2/ml due to significant decrease in water droplet size. It therefore solves the problem of nonaqueous enzyme reaction systems. The lowered stability, altered specificity of enzymes [8, 9], and mass transfer limitation of two-phase systems can be remedied. However, surfactants are extremely difficult to separate from products, thus limiting the application. DMS does not have such a problem because 2-propanol can be easily removed [3, 12, 16]. There are only two DMS systems reported, which use hexane and toluene as the organic solvent media in conjunction with water and 2-propanol. More than 30 water-soluble enzymes, including horse radish peroxidase (HRP) and laccase, have been reported [1, 13, 20]. In our study, urushiol, water, and 2-propanol were used to create DMS. The formation of micro-emulsion was studied by conductance measurements. The study revealed the phase changes of the mixture, as the molar ratio of 2-propanol increased. The existence of the micro-emulsion phase was again verified by the dynamic light scattering method to measure the mean hydrodynamic radius. In other DMS and micro-emulsion systems, substrates were added to the solvent to be reacted by the enzyme, which were present in the micro-droplet of water. In this study, a novel concept of using a natural oil urushiol, not only as a hydrophobic solvent but also as a substrate, is proposed. Recently, there have been many studies on the enzymatic modification of fats and oils for production of high-value products [7]. Our study suggests that other fats and oil could be used to create a similar DMS, and these systems would not have problems of enzyme stability, enzyme specificity, reaction surface, and surfactant removal associated with organic solvent enzyme reaction systems. Our system can provide a new, viable alternative for such applications.

MATERIALS AND METHODS

Urushiol Purification

Urushiol was purchased directly from a farm in Wonju, Korea (collected July 4th, 1999) to ensure purity and consistency of the data. One liter of urushiol was mixed with acetonitrile at 1:1 ratio and it was stirred for 20 min. The solution was centrifuged at 3,000 rpm (Hitachi, Japan) for 15 min. The solution separates into 3 distinct phases, which includes the urushiol-containing top phase, gum-containing middle phase, and clear blue water phase with soluble polysaccharides and enzymes. Each phase was separated and collected. Pure urushiol was obtained by evaporating the acetonitrile using a rotary evaporator (Eyla, Japan) followed by double vacuum distillation.

Conductance Measurements

2-Propanol was purchased from Sigma Chem. Co. and distilled water was further purified using a small scale distillation unit. The conductance was measured by an electrode type conductometer (DIK, Korea). Urushiol and distilled water (10% v/v) were mixed at 25°C, and the conductance was measured after appropriate amounts of 2-propanol were added.

Dynamic Light Scattering

For dynamic light scattering (DLS) experiments, a helium-neon ion laser (Brookhaven, USA) was used as the light source. The operation wavelength was 633 nm at a constant output of 10 mV. The sample was positioned in a xylene bath mounted on a goniometer and thermostated at 25°C by a standardized corellator (Brookhaven, U.S.A.). All measurements were made at a scattering angle of 90°, and the intensity auto-correlation functions were analyzed using a cumulant method. All samples were passed through 0.2 μm pore Gelman PTFE filters to remove dust particles.

Laccase Purification

Purification of laccase was carried out by a modified Reihnhammar method [19]. The enzyme in water phase from the acetonitrile separation method was used rather than filtering an acetone powder mixture, in order to avoid the difficulties arising from filter clogging. The rest of the protocol included the use of CM-Sephadex C-50 and DEAE A-50 chromatographies and related materials.

Laccase Activity Assay

A spectrophotometer (Kontron, Switzerland) was used to measure absorbance change at 530 nm. Syringaldezine purchased from Sigma Chem. Co. was used as the substrate at 0.5 mM concentration. The laccase purified by the modified Reinhammar method described above was used. The absorbance was measured by adding 2 ml of 0.04 M phosphate buffer (pH 6), 0.9 ml of 0.5 mM syringaldezine, and 0.1 ml of the enzyme suspension. When the specific activity was measured in the 2-propanol system, 0.08 M potassium phosphate buffer of pH 6 was used. The substrate and the enzyme concentrations were set to be identical, in order to verify the differences in enzyme activity.

Urushiol Polymerization Using Bubble Column Reactor

A 100-ml bubble column reactor with 3 cm diameter and 20 cm length was constructed, as shown in Fig. 3. In order to minimize the loss of volatile 2-propanol, a 15-cm compact condenser unit immersed in an ice bath was utilized. The airflow rate was set at 1.5 l/min and the temperature was at 30°C. The reaction mixture comprised of 56% (v/v) urushiol, 34% (v/v) 2-propanol, and 10% (v/v) enzyme extract, which was found to make micro-emulsion. The reaction was conducted for 15 h and the viscosity was measured using Rheometrics mechanical spectroscopy (Rheometrics, U.S.A.). It must be noted that the urushiol used was partially purified from extract using acetonitrile.

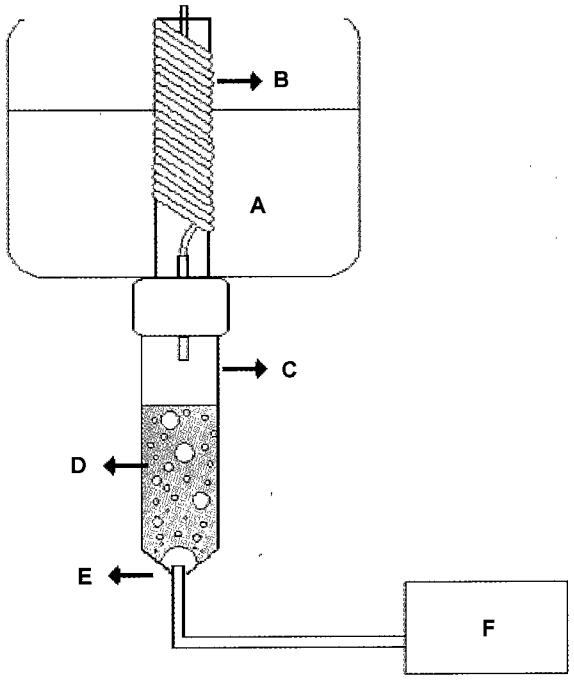

Fig. 3. Schematic diagram of the DMS bubble column reactor for urushiol polymerization.
A (Ice bath), B (Condenser), C (100 ml reactor tube), D (Urushiol/water/2-propanol mixture), E (Air hose), F (Flux).

The enzyme extract was prepared from the bottom phase of the acetonitrile separation, and was diluted 1/100 with distilled water to be used. For a comparative study, other organic solvents including acetonitrile, THF, and ethanol were also used to react with urushiol under the same conditions.

RESULTS AND DISCUSSION

Micro-Emulsion Formation and Conductance Change

Researches on the detergentless micro-emulsion system using 2-propanol and water with hexane and toluene revealed that the micro-emulsion condition could be detected by measuring the change in conductivity as the 2-propanol concentration was varied [12, 16]. In normal water in oil emulsions, water droplets exist as a result of forced agitation, resulting in the temporary creation and dispersion of unstable water droplets. The solution is turbid and the water droplets ultimately aggregate among themselves, resulting in two distinct phases. However, in the case of a micro-emulsion, an optically clear solution results and the phase separation does not occur. Preliminary studies on DMS revealed that as the molar ratio of 2-proplanol rose, the size of the water droplet decreased and the dispersity increased. The level of dispersity dictates the conductance, and hence it rises as the 2-propanol concentration is slowly raised. However, as the 2-propanol reaches a certain mole fraction, where formation of micro-emulsion is favored, the size of the water droplets decreases dramatically. It results in high dispersity and, therefore, conductivity of the solution rises abruptly. This condition is sustained until 2-propanol concentration becomes too high for it to function properly. Interestingly enough, it begins to act as a polar organic solvent. At this juncture, the water droplets are broken down and the water molecules exist as unstable aggregates, and further addition results in complete mixing of the three components. Due to the inherently low conductivity of 2-propanol, further addition results in a slow decrease in conductivity.

In our study, a representative trend of the conductance change for micro-emulsion formation was observed when pure urushiol was used as the oil phase. As seen in Fig. 4a, an abrupt rise in conductivity was observed when the mole fraction of 2-propanol reached to 0.4. The conductivity continued to rise until the mole fraction reached around 0.44. This range can be assumed to be a micro-emulsion phase which is called L2 phase. It was easy to identify, as the turbid emulsion mixture suddenly became optically clear. However, urushiol is an oil that has a benzene ring with two hydroxyl groups and a hydrophobic hydrocarbon tail, which can be viewed as being functionally similar to a surfactant. In order to dismiss a possibility that urushiol itself, rather than 2-propanol, was responsible for the phenomenon, additional experiments were performed using n-propanol

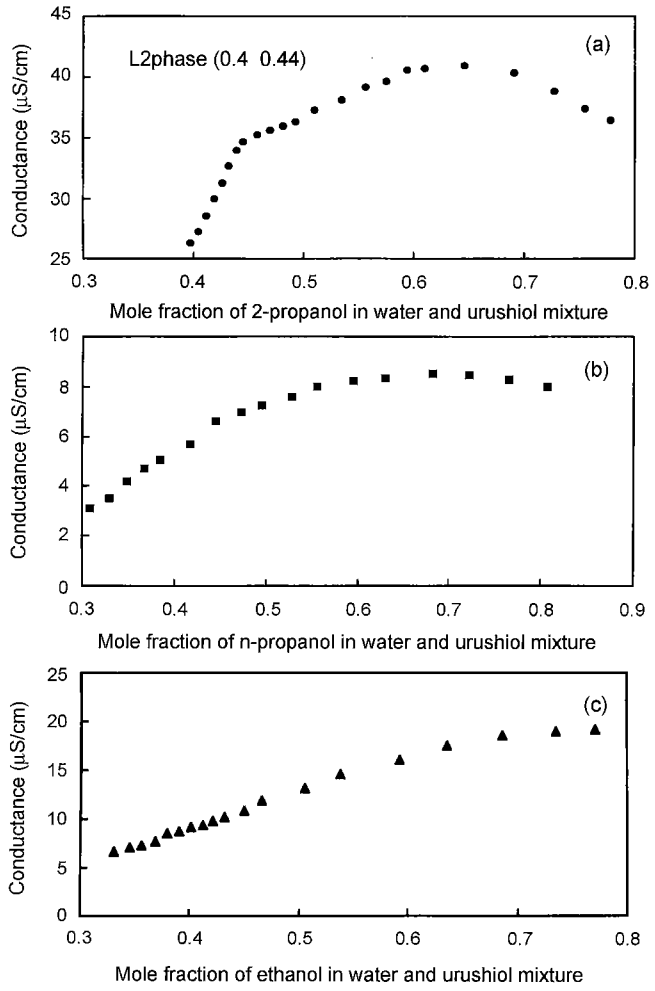

Fig. 4. Conductance measurements of solutions.
(a) 2-Propanol, (b) n-propanol, and (c) ethanol were added in increments to the original solution of 10% (v/v) water and 90% (v/v) urushiol.

and ethanol for the comparative study. These polar solvents were chosen for their chemical and functional similarities. Being an isomer, n-propanol showed similar conductivity levels at differing concentrations. However, the curve was smooth, indicating the normal dilution and dissolving kinetics that could be seen when polar organic solvents were added to 'water in oil' mixtures (Fig. 4b). In the case of ethanol, the inherent conductivity was relatively high, therefore yielding a smooth curve that continued to rise (Fig 4c). It was quite possible that the other solvents did not form micro-emulsions. Both the characteristic conductance and the comparative study confirmed the formation of micro-emulsion.

Micro-Emulsion Formation and Dynamic Light Scattering

The water droplets are stable only in the range where micro-emulsion is formed. The samples were agitated and placed in a thermostated xylene well for dynamic light scattering analysis at 25°C. When 2-propanol concentration was low, the solution appeared turbid, therefore, analysis

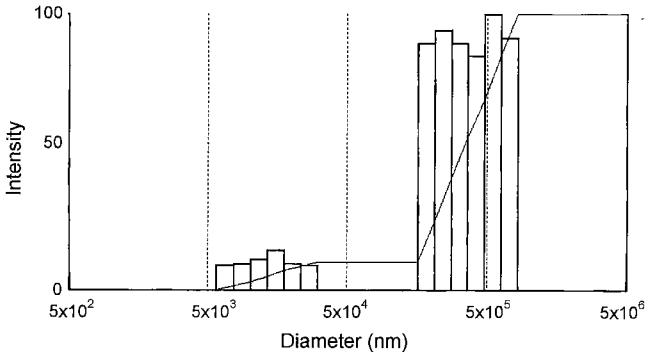

Fig. 5. DLS measurement showing the observed peak of mean water droplet diameter at the transitionary phase where unstable separation of smaller micro-droplets begins to appear.

was not possible. The dynamic nature of the water droplets caused rapid change in the hydrodynamic radius, due to aggregation, as a function of time. The 2-propanol concentration, at which micro-emulsion phase begins, can be detected because separation of radius distribution occurs. When the solution was in normal emulsion state, a widely ranging radius distribution was detected that had an average diameter in the thousands to tens of thousands of nanometers. As the mole fraction of 2-propanol approached 0.4, a transitionary phase was seen (Fig. 5), where separation of large globules of water and smaller droplets began to appear. However, at this molar range, micro-droplets were not very stable and they seemed to disappear after a couple of hours. When more 2-propanol was added in small increments, concentrated peaks of micro-emulsion were detected (Fig. 6). The increasing 2-propanol concentration resulted in rapid decrease in the hydrodynamic radius (Fig. 7). The graph represents the change in the mean water droplet size and it is easy to see that this abrupt change aproached complete mixing as the mole fraction of 2-propanol was further increased. In the secondary transitional phase, where the droplets were supposedly broken down and no longer able to hold its form, unstable peaks were once again detected. Further addition of 2-propanol resulted in a solution in

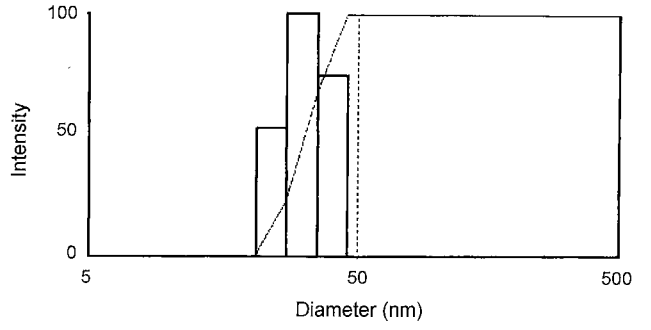

Fig. 6. DLS measurement showing the mean diameter of water droplets at the mole fraction of 0.41.
Near singular peaks of radius of 46 nm can be seen.

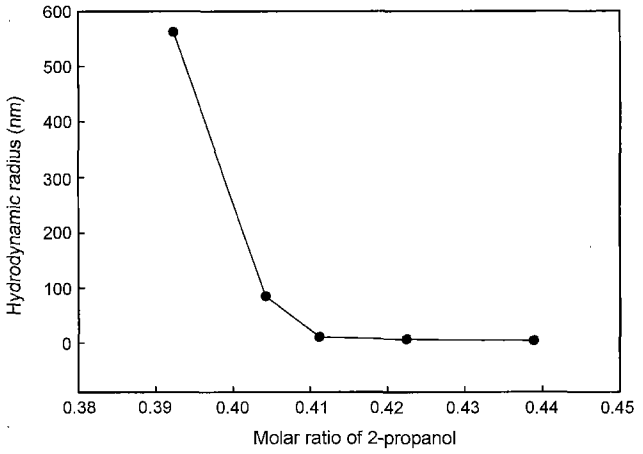

Fig. 7. The mean hydrodynamic radius of water droplets at differing molar ratios of 2-propanol, which shows rapid decrease in the diameter.

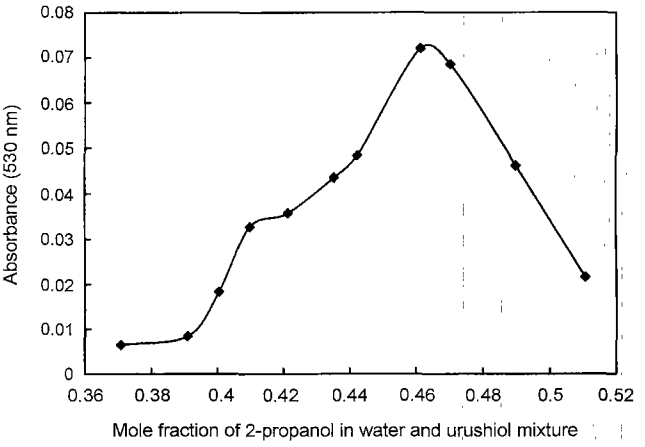

Fig. 8. Relative enzyme activity of laccase analyzed by measuring absorbance at 530 nm using syringaldezine as substrate in a DMS system [◆ Laccase activity (abs/min)].

which the hydrodynamic radius was no longer detectable. The secondary transitionary phase occurred when the mole fraction of 2-propanol was around 0.44. This was in accordance with conductance data.

Laccase Activity Characterization in DMS System

The characterization of laccase activity in a DMS system was studied. In our study, urushiol was used as both solvent and substrate, therefore direct measurement of urushiol polymerization by laccase would have been ideal. However, urushiol has a phenol group and the polymerization reaction results in a dark brown color from bright yellow. Furthermore, the reaction was very slow and measurement of the enzyme activity by absorbance change was not possible. In order to have significant data, the reaction had to be carried out for hours, however, the mass transfer problem of oxygen needed by laccase led to incoherent data. Therefore, syringaldezine was used as the substrate to minimize the addition effect of non-urushiol substrate. In our experiment, a 0.5 mM solution in 2-propanol was prepared and the solution was added to samples that resulted in set concentration of 0.0726 mM. Also, the substrate had to be highly hydrophobic so that it would mostly stay in the solvent phase and not in the water droplet phase. Syringaldezine was practically insoluble in water, so we were able to gain clear results.

The laccase activity in the urushiol, water, and 2-propanol system gave interesting results. Laccase solution of 0.247 A/min was prepared in 0.8 mM potassium phosphate buffer of pH 6 and added to the system such that laccase activity was 0.0120 A/min. It was very important that the concentrations of the enzyme and the substrate in the samples were the same, so that meaningful comparative data could be made possible. The change in laccase activity was measured and analyzed between 2-propanol mole fractions of 0.37 to 0.51 (Fig. 8). When the 2-propanol ratio was around 0.4, where micro-emulsion was expected to occur, the enzyme activity rose sharply and it corresponded with the expected data of the conductance and DLS study. It suggests that the increased reaction surface enabled laccase to function more effectively. In the conductance and DLS study, 0.44 was the point where emulsion was thought to begin breaking up into water aggregates. However, the activity continued to rise even more abruptly until around 0.46. Over this point, the activity fell sharply. There are two possible explanations for these phenomena. First, it is possible that laccase and substrate affected the DMS system and broadened the range where it can hold its form. The laccase is roughly round in its form and one can visualize a thin water layer surrounding and packaging the enzyme, using the protein's hydrophilic surface as a support. Another possibility is that the substrate syringaldezine and the salt present in the buffer somehow played a role in holding the system together. However, it is clear that the heightened activity shown between the mole fraction of 0.44 and 0.46 was functioning as a type of micro-emulsion system, but not an organic solvent system. If this mole fraction area represented an organic solvent system, then the rise in activity should gradually increase after 0.44 and taper off at a saturation point. Our data showed rapid decrease in the enzyme activity after 0.46. When the mole fraction of 2-propanol was larger then 0.46, the fraction of 2-propanol was large enough so that it would completely dissolve water and become an organic solvent system, where no great differences in mass transfer or effect to the enzyme can occur. This study showed the existence of an optimal zone where the relative enzyme activity was much higher. However, more detailed study on the broadening effect is needed.

Urushiol Polymerization Using Bubble Column Reactor

Laccase polymerization of urushiol was conducted in a small-scale bubble column reactor to provide oxygen

needed by laccase to function and to achieve mixing. The viscosity was measured as a function of time to study the increase in urushiol polymerization level. Traditional enzymatic polymerization step (pretreatment method called Gurome) raises the viscosity of urushi sap from 1,500 cP to around 3,000 cP. This is possible only when full laccase in the sap is available. The level of laccase is thought to be insufficient in many cases and there are many patents and studies on adding additional laccase from fungal or mushroom sources. Our goal was to create an operational process that could react urushiol to around 3,000 cP with as little laccase as possible, so that it could be used to create traditional lacquer as well as find application in the industrial area. In our study, the diluted (1/100) laccase and soluble polysaccharide containing water phase, separated using the newly developed acetonitrile method, was used to polymerize urushiol. Enzyme assay of the laccase present in the diluted solution was 0.0026 A/min. We were able to raise the viscosity from 142 cP (initially low due to lack of non-urushiol components such as gum) to around 3,000 cP in 14 h (Fig. 9). However, it was interesting to find that addition of purified laccase did not significantly extend the reaction time. In conjunction to this experiment, organic solvent systems with an equal condition of adding 34% (v/v) to 10% (v/v) water and 56% (v/v) urushiol were tested for a comparative study. The solvents used were acetonitrile, THF (tretrahydrofuran), and ethanol. Water was used as the control. The results showed that the 2-propanol system had the highest level of polymerization. In the case of other solvents, the effect of the solvents on the enzyme stability seemed to be the main factor when raising the viscosity. Khmelnitsky *et al.* [14] have studied the process of reversible denaturation of various proteins, including laccase, by organic solvents. Ethanol had the least effect on laccase stability and it is very similar to 2-propanol. Acetonitrile was the second and THF was known to be the worst for enzyme stability. The rise in viscosity of these systems followed the trend stringently. In the control, where water was added instead of an organic solvent, the reaction level was as low as that of THF. The solution during the reaction was highly turbid and serious separation between water and urushiol seemed to occur. It is likely that the limitation of a reaction surface, where laccase and urushiol can react, is the cause of the low reaction level. 2-Propanol is also a mild solvent, closely resembling ethanol, but it produced significantly higher viscosity levels.

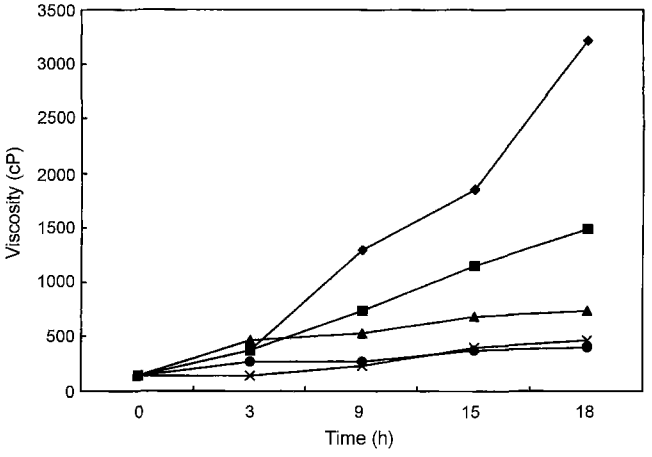

Fig. 9. Increase in viscosity of urushiol polymerization in the bubble column reactor with 32% (v/v) addition of various organic solvents.
× water, ● THF, ▲ acetonitrile, ■ ethanol, ◆ 2 propanol.

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