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# Lipase-Catalyzed Solvent-Free Transesterification of Wood Sterols

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## Abstract

Eighteen commercial lipase preparations, either immobilized or crude enzyme powders, were screened for the transesterification of wood sterols. The reactions were carried out in a solvent-free system, at the optimum temperature of the enzyme preparations as reported by the manufacturer and at the pressure of 2 mbar, with 5 or 10% in weight of the enzyme relative to the wood sterol content of the reacting mixture. Methyl esters of sunflower fatty acids were used as transesterifying agent. Of all the enzymes assayed, only Lipase TL from *Pseudomonas stutzeri* PL-836 (Meito Sangyo) exhibited any significant transesterifying capacity, 85 and 95% of conversion after 2 and 8 h of reaction, respectively, when 10% in weight of enzyme was used.

**Index Entries:** Enzymatic transesterification; lipase; sterols; steryl esters; solvent-free system.

## Introduction

Sterols and stanols and their fatty esters are useful cholesterol-lowering agents and as such are currently in high demand as nutraceutical food ingredients *(1–3)*. Steryl or stanyl esters are preferred to free sterols or stanols, owing to their better miscibility with fatty foods such as margarine.

Steryl and stanyl esters can be made from free sterols or stanols by organic synthesis using different catalysts *(4)*. The many advantages of

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organic synthesis, including a process for the enzymatic esterification or transesterification of sterols with fatty acids or with fatty acid esters in aqueous media or in media containing water-saturated organic solvent, are discussed elsewhere *(5)*. However, the process has several setbacks as well.

The presence of free water in the reacting mixture may favor hydrolysis over synthesis, as can be seen from the figures in the examples shown.

Additionally, some lipases do not exhibit any activity in water-saturated organic solvents *(6)*. Furthermore, the utilization of organic solvents might not be compatible with the elaboration of nutraceutical food ingredients. Therefore, the interest in studying lipase-catalyzed synthesis in nonaqueous or solvent-free systems is strongly motivated, and there are several recent reports in the literature *(7–9)*. To carry out the transesterification of wood sterols, which are solids at room temperature, without any organic solvent to keep them in dissolution, the transesterifying reactant, i.e., the fatty acid methyl esters (FAMEs), should be acting as solvent. The solubility of the wood sterols in FAMEs at <40°C is very low, but above this temperature the FAMEs are capable of solubilizing fair amounts of sterols. Actually, the solubilities of sterols in FAMEs at 40, 50, and 60°C are 15, 20, and 25% by weight, respectively, which is enough for a production-oriented process.

Lipase-catalyzed solvent-free transesterification of sterols and stanols has been reported elsewhere *(10–12)*. There are, however, important differences between these reports and our work concerning the size or scale of the reaction system and the enzyme-to-substrate ratio utilized.

We studied the transesterification of wood sterols with lipases under operating conditions readily amenable to scale-up for large-scale production, utilizing at the same time a low enzyme-to-substrate ratio to allow for commercial feasibility of an eventual large-scale production. This article reports the results obtained on the transesterification of wood sterols with methyl esters of sunflower oil fatty acids with 18 commercially available lipase preparations.

## Materials and Methods

### *Wood Sterols*

Wood sterols of the composition shown in Table 1, consisting of a mixture of sterols and stanols derived from black liquor soaps, a byproduct of pine wood pulping by the kraft process, were kindly provided by Härting S.A., a local chemical manufacturing company that manufactures wood sterols by means of a proprietary technology *(13)*.

### *Lipases*

The lipase preparations used are provided in Table 2.

Table 1
Composition of Wood Sterols

| Sterol | Percentage in weight |
|---|---|
| β-sitosterol | 68 |
| β-sitostanol | 20 |
| Campesterol | 7 |
| Campestanol | 2 |
| Stigmasterol | <1 |
| Others | 2 |

### *Methyl Esters of Sunflower Oil Fatty Acids (FAMEs)*

FAMEs were prepared from commercially available sunflower oil fatty acids as follows: Ten liters of technical-grade methanol, 313 g of concentrated sulfuric acid, and 10.3 kg of fatty acids were loaded under slight nitrogen flux in a 40-L stainless steel agitated reactor provided with electrical heater, refrigeration coil, loading funnel, column, condenser, Dean-Stark separator, bottom valve, nitrogen injector, and vacuum connection for distillation at reduced pressure. Nitrogen flux was interrupted, and the mixture was refluxed at 77°C for 10 h with periodic monitoring of the acid number. When the acid number reached 6.4, methanol was distilled off until 90% of the initial amount of methanol was recovered. The mixture was cooled down to 60°C and diluted with 7 kg of hexane. Remaining acidity was neutralized with 8 kg of an aqueous solution of technical- grade sodium carbonate at 10%. The aqueous phase was eliminated, and the organic phase was washed three times with 1 kg of a water:methanol (1:1) mixture to pH 6.8. The neutral organic phase was desolventized at 95°C and 25 mbar, obtaining 9.64 kg of a brownish mixture of methyl esters of sunflower oil fatty acids.

This mixture was further purified by distillation in a short-path KDL-5 unit (UIC GmbH) at 160°C and 1 mbar, obtaining a slightly yellow product of purified FAMEs.

### *Reaction of Transesterification*

Reactions were carried out in a 2000-mL Schott-Duran round-bottomed, flat flange reaction vessel with flat flange lid, provided with four standard ground necks, containing 500 mL of FAMEs saturated with wood sterols and 5 or 10% in weight of enzyme preparation relative to the wood sterol content of the reacting mixture. The enzyme preparations were previously incubated overnight in an environment of constant water activity of 0.75 and 25°C (saturated NaCl solution).

One of the reactor outlets was connected to a vacuum pump (TRIVAC B D 16B) keeping the pressure at 2 mbar in the reactor during the reaction. Stirring was done with a magnetic stirrer. Total reaction time was 2 h.

Table 2
Lipase Preparations Used for Transesterification of Wood Sterols

| Lipase | Derived from | Manufacturer | Characteristics | Lipolytic units/g[a] |
|---|---|---|---|---|
| Lipase QLG | *Alcaligenes* sp. | Meito Sangyo | Immobilized enzyme on granulated diatomaceous earth | 20,000 |
| Lipase QLC | *Alcaligenes* sp. | Meito Sangyo | Immobilized enzyme on diatomaceous earth | 20,000 |
| Lipase SL | *Pseudomonas (Burkholderia) cepacia* SL-25 | Meito Sangyo | Crude enzyme powder | 45,000 |
| Lipase UL | *Rhizopus* sp. Q 119 | Meito Sangyo | Crude enzyme powder | 50,000 |
| Lipase TL | *Pseudomonas stutzeri* PL-836 | Meito Sangyo | Crude enzyme powder | 100,000 |
| Lipase PLG | *Alcaligenes* sp. | Meito Sangyo | Immobilized enzyme on granulated diatomaceous earth | 15,000 |
| Lipase PLC | *Alcaligenes* sp. | Meito Sangyo | Immobilized enzyme on diatomaceous earth | 15,000 |
| Lipase ALG | *Achromobacter* sp. | Meito Sangyo | Immobilized enzyme on granulated diatomaceous earth | 30,000 |
| Lipase ALC | *Achromobacter* sp. | Meito Sangyo | Immobilized enzyme on diatomaceous earth | 300,000 |
| Lipolyve AN | *Aspergillus niger* | Lyven | Water-soluble powder | |
| Lipolyve CC | *Candida cylindracea* | Lyven | Water-soluble powder | |
| Lipolyve R | *Rhizopus oryzae* | Lyven | Water-soluble powder | |
| Lipase PS-D "Amano" I | *Pseudomonas cepacia* | Amano | Immobilized enzyme on diatomaceous earth | 701 |
| Lipase PS-C "Amano" I | *Pseudomonas cepacia* | Amano | Immobilized enzyme on ceramic particles | 1590 |
| Lipase PS-C "Amano" II | *Pseudomonas cepacia* | Amano | Immobilized enzyme on ceramic particles | 866 |
| *Validase*® Fungal Lipase 8000 | *Rhizopus oryzae* (ATCC 1996) | Valley Research | Crude enzyme powder | 80,000 |
| Lipolase 100 T | *Humicola (Thermomyces) lanuginosa* | Novo Nordisk | Immobilized | 100 KLU/g |
| Lipozyme IM | *Rhizomucor miehei* | Novo Nordisk | Immobilized | 7.1 BAUN/g |

[a]As reported by the manufacturer.

With Lipase TL, two additional 8-h runs were conducted as well, the first at 2 mbar and the second at atmospheric pressure. At 1-h intervals, the vacuum was broken so that samples could be taken. The sampling procedure (breaking the vacuum, taking a sample, and restoring the vacuum) took only 30–60 s.

The samples (<0.5 mL each time), after removing eventual residual enzyme, were analyzed for free sterols and stanols, and the corresponding conversion $X$ at time $t$ was calculated as follows:

$$X = (C_0 - C)/C_0 \cdot 100$$

in which $C_0$ is the concentration of free sterols and stanols in the reactant mixture at time 0, and $C$ is the concentration of free sterols and stanols at time $t$. For each enzyme preparation two independent assays were done.

### *Analytical Methods*

The method of analysis of free sterols and stanols is very reliable and reproducible and is described in some detail.

### *Preparation of Sample*

One hundred milligrams of sample was dissolved in tetrahydrofurane in a 25-mL volumetric flask, and 500 μL of this solution was introduced into a silanization tube. Next, 50 mg of 5β-cholestan-3α-ol was dissolved in *n*-propanol in a 100-mL volumetric flask. Then, 500 μL of this solution was added to the silanization tube, and the solvent was evaporated with gentle heating under nitrogen. Next, 300 μL of bis (trimethylsilyl) trifluoroacetamide and 300 μL of pyridine were added, the mixture was heated at 70°C for 10 min, and then the liquid phase was evaporated under a nitrogen atmosphere. Finally, the residue was dissolved in 500 μL of tetrahydrofurane.

### *Analysis*

Analysis of free stanols and sterols in the samples treated as described was carried out (0.5-μL sample injection) using a Hewlett-Packard HP 6890 series 2 chromatograph provided with an HP-5 capillary column (30m long, 0.32-mm in diameter, and a 0.25-mm film) with oven and injector temperatures of 300 and 320°C, respectively; helium as carrier gas at 0.92 mL/min with a 60:1 split; and a 15-min program.

## Results

Table 3 shows the conversion of wood sterols to wood steryl esters catalyzed by the enzymatic preparations utilized, at the temperatures indicated after 2 h of reaction. The results show that only Lipase TL followed by Lipase SL exhibited any significant conversion of the wood sterols to the corresponding steryl esters. Therefore, further, longer runs were carried out with Lipase TL to study the kinetics of the reaction.

Table 3
Conversion of Wood Sterols to Fatty Steryl Esters
After 2 h of Reaction Time, 2 mbar, and 5% in Weight
of Enzyme Relative to Wood Sterols

| Lipase | Temperature (°C) | Conversion (%)[a] |
|---|---|---|
| Lipase QLG | 60 | 9 |
| Lipase QLC | 60 | 12 |
| Lipase SL | 70 | 18 |
| Lipase UL | 45 | 1 |
| Lipase TL | 50 | 50 |
| Lipase PLG | 40 | 0 |
| Lipase PLC | 40 | 0 |
| Lipase ALG | 45 | 0 |
| Lipase ALC | 45 | 0 |
| Lipolyve AN | 40 | 0 |
| Lipolyve CC | 40 | 2 |
| Lipolyve R | 40 | 0 |
| Lipase PS-D "Amano" I | 50 | 1 |
| Lipase PS-C "Amano" I | 50 | 7 |
| Lipase PS-C "Amano" II | 50 | 0 |
| *Validase*® Fungal Lipase 8000 | 40 | 0 |
| Lipolase 100 T | 45 | 4 |
| Lipozyme IM | 60 | 0 |

[a]Average of two independent assays rounded to one significant figure.

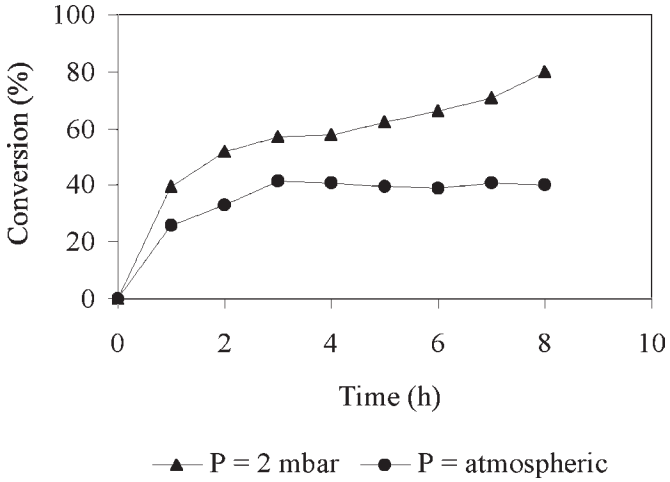


Fig. 1. Transesterification reaction between wood sterols and FAME with Lipase TL at two different pressures. (▲) 2 mbar, (●) atmospheric pressure (50°C, 5% in weight of the enzyme relative to the wood sterol content of the reacting mixture).

The course of the transesterification reaction with Lipase TL at two different pressures is compared in Fig. 1. Figure 2 shows the course of the reaction at 2 mbar.

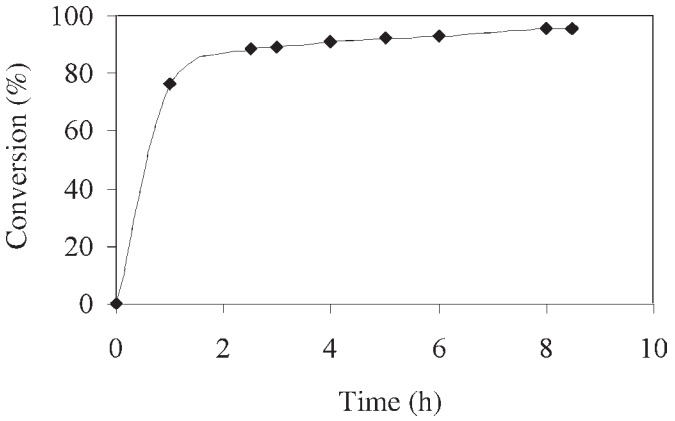


Fig. 2. Transesterification reaction between wood sterols and FAME with Lipase TL (60°C, 2 mbar, 10% lipase in weight relative to wood sterols).

## Discussion

As can be observed from the data of Table 3, of the 18 different commercial preparations of lipase assayed, most of them did not catalyze the transesterification of wood sterols under the conditions of the assay, some did catalyze the reaction at a very low extent, but significant conversion of wood sterols to steryl esters was obtained only with Lipase TL.

Table 3 also shows that Lipozyme IM did not catalyze the transesterification of the mixture of wood sterols to any significant extent. This result is apparently in sharp contrast with results reported elsewhere *(11)* and indicates a nearly complete conversion of sterols to their respective fatty acyl esters after 32 h of reaction time at 80°C and an enzyme-to-substrate ratio over 1 (w/w), (100 μmol, about 40 mg of sitostanol/50 mg of enzyme) in the presence of Lipozyme IM. In our case, the reactions were carried out at 60°C for 2 h using an enzyme-to-substrate ratio of 0.05 or 0.10. These very significant differences in the conditions of reactions could possibly account for the different results obtained with Lipozyme IM. However, note that at short reaction times and at 60°C, the conversion with Lipozyme IM even at an enzyme-to-substrate ratio as high as 1 (w/w) was negligible, as shown in Weber et al. *(11)*. Consequently, the differences reported concerning the sterol transesterifying activity of Lipozyme IM are not conflictive.

In the reactions with Lipase TL, as shown in Fig. 1, after 8 h of reaction the conversion of sterols to steryl esters was about 95% and still increasing with time. Nonetheless, a mixture of 95% steryl esters and 5% free sterols is easily miscible with margarine, and therefore it is not strictly necessary to further increase conversion, though it can be done.

The mixture of nonreacted FAMEs can be easily removed from the reacted mixture by low-pressure distillation, either in a short-path distillation column or in a thin-film evaporator. To this end, Lipase TL that is sold

as a crude enzyme powder should be first immobilized, before attempting to optimize the process. Our current work is addressing this problem.

As Fig. 2 shows, the pressure, as expected, had an important effect on the overall conversion. This might be explained by the continuous removal of the methanol formed during the reaction, and perhaps also by the removal of possible excess humidity from the reactant mixture.

It is also worth mentioning that neither of the lipases assayed catalyzed the direct esterification of wood sterols with free fatty acids under the conditions of our study.

## Conclusion

The feasibility of preparing wood sterol esters in a solvent-free medium by means of lipase-catalyzed transesterification of the wood sterols with FAMEs with low enzyme-to-substrate ratio and relatively short reaction times was demonstrated. Conversion can be nearly complete under suitable conditions. The performance of Lipase TL for the transesterification of wood sterols is outstanding and possibly the best among all those reported in the literature. Therefore, the enzymatic, solvent-free esterification of sterols with Lipase TL might become an economically attractive alternative method to the chemical esterification of these compounds.

## Acknowledgment

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