that the very desired product, simvastatin, competes for the LovD enzyme, significantly impeding the overall net rate of acylation (*id.*).

[0010] The LovD enzyme is also highly prone to mis-folding and aggregates when over-expressed in *E. coli*, making even whole-cell biocatalysis systems less than ideal for commercial production (Xie *et al.*, 2009, Biotech. Bio Eng. 102:20-28).

[0011] In an effort to increase LovD solubility without loss of catalytic activity, mutants of wild-type *A. terreus* LovD have been studied. Replacing the cysteine residues at positions 40 and 60 (Cys40 and Cys60) with alanine residues yielded improvements in both enzyme solubility and whole-cell biocatalytic activity (*id.*). Further mutagenesis experiments converting these two residues to small or polar amino acids showed that Cys40→Ala ("C40A") and Cys60→Asn ("C60N") mutations are the most beneficial, yielding 27% and 26% increases, respectively, in whole-cell biocatalytic activity (*id.*). When combined, these mutations proved additive, with the C40A/C60N double mutant exhibiting approximately 50% increases in both solubility and whole-cell biocatalytic activity.

[0012] Despite their improved properties, these LovD mutants are unsuitable for large scale production of simvastatin in cell-free systems. Additional variants or mutants of wild-type *A. terreus* LovD enzymes that exhibit improved properties as compared to the wild-type and/or known mutants would be desirable.

4. SUMMARY

[0013] As discussed above, the *LovD* gene of *A. terreus* encodes an acyl transferase (hereinafter called "LovD polypeptide," "LovD enzyme," "LovD acyltransferase" or "LovD") capable of converting monacolin J, the hydrolysis product of the natural product lovastatin, to simvastatin. The inventors of the present disclosure have discovered that LovD polypeptides including mutations at certain residue positions exhibit improved properties as compared to the wild-type LovD polypeptide produced by *A. terreus* (SEQ ID NO:2).

[0013a] In one aspect, there is provided a variant LovD polypeptide having at least two-fold greater catalytic activity than the wild-type *A. terreus* acyltransferase of SEQ ID NO:2, wherein the variant LovD polypeptide comprises the amino acid sequence of SEQ ID NO:2 that includes the following two mutations: L174F and A178L, and optionally from about 1 to 30 additional mutations and optionally from about 1 to 20 additional conservative mutations.

[0014] Accordingly, in one ~~aspect~~embodiment, the present disclosure provides variant LovD polypeptides that have one or more improved properties as compared to wild-type LovD polypeptide from *A. terreus* (SEQ ID NO:2). Generally, the LovD variants include one or more mutations at selected positions that correlate with one or more improved properties, such as increased catalytic

residue positions (L174F and A178L) exhibited approximately two-fold greater activity than wild-type LovD, and a specific variant including mutations at six residue positions (SEQ ID NO:120) exhibited approximately 12-fold greater catalytic activity than wild-type LovD, one specific variant including 28 mutations (SEQ ID NO:114), one specific variant including 29 mutations (SEQ ID NO:116) and one specific variant including 32 mutations (SEQ ID NO:118) each exhibited approximately 1550-fold greater catalytic activity than wild-type LovD. Table 1 discloses numerous variant LovD polypeptides exhibiting from about 10- to about 1550-fold greater activity than the wild-type LovD polypeptide of SEQ ID NO:2. Using these exemplary specific variant LovD polypeptides, additional LovD variant polypeptides having greater than about 10-, 20-, 30-, 40-, 50-, 60-, 70-, 80-, 90-, 100-, 150-, 200-, 300-, 400-, 500-, 600-, 700-, 800-, 900-, 1000-, 1100-, 1200-, 1300-, 1400-, 1500-, 1600-, 1700-fold, or even greater, activity than the wild-type LovD polypeptide of SEQ ID NO:2 can be readily obtained.

[0025] The specific variant LovD polypeptides of Table 1 can also be used to readily obtain variant LovD polypeptides that have specified combinations of improved properties.

[0026] The various variant LovD polypeptides described herein may also optionally include one or more conservative mutations in addition to the mutations described above. Typically, such optional conservative mutations will not comprise more than about 20%, 15%, 10%, 8%, 5%, 3%, 2%, or 1% of the overall sequence. Without intending to be bound by any particular theory of operation, it is believed that the amino acid at position 76 may be involved in catalysis. Mutations at this residue position should be preferably avoided. It is also believed that the amino acids at position 79, 148, 188 and/or 363 may contribute to catalysis. In some embodiments, variant LovD polypeptides that include optional conservative mutations will contain from 1 to 20 such mutations. In additional embodiments, variant LovD polypeptides include truncated polypeptides wherein 1 to 15 amino acids may be omitted from the N-terminus and 1 to 6 amino acids may be omitted from the C-terminus.

[0026a] In one aspect, there is provided a polynucleotide encoding a variant LovD polypeptide as defined above.

[0027] In another ~~aspect~~embodiment, the disclosure provides polynucleotides encoding the variant LovD polypeptides described herein. In some embodiments the encoding polynucleotides are part of an expression vector comprising one or more control sequences suitable for directing expression of the encoding sequence in a host cell. In some embodiments, the expression vector is suitable

for expressing variant LovD polypeptides in a bacterium, such as but not limited, to an *E. coli*, and includes a promoter sequence, such as a *lac* promoter sequence, operably linked to the encoding sequence. In such polynucleotides the codons of the encoding sequence can be optimized for expression in a particular host cell of interest.

[0027a] In one aspect, there is provided a host cell comprising a polynucleotide encoding a variant LovD polypeptide as defined above.

[0028] In yet another ~~aspect~~embodiment, the present disclosure provides host cells comprising a variant LovD encoding polynucleotide or expression vector. In some embodiments, the host cell is a bacterium such as but not limited to *E. coli*. The host cells can be used to produce crude or purified preparations of specific variant LovD polypeptides, or, alternatively, they can be used as whole-cell preparations in the methods described herein to produce simvastatin and huvastatin.

[0028a] In one aspect, there is provided a method of making a variant LovD polypeptide comprising culturing a host cell as defined above under conditions in which the variant LovD polypeptide is expressed and optionally recovering the variant LovD polypeptide.

[0029] The variant LovD polypeptides described herein esterify the C8 hydroxyl group of monacolin J in the presence of an α-dimethylbutyryl thioester co-substrate to yield simvastatin.

[0029a] In one aspect, there is provided a method of making simvastatin comprising contacting a monacolin J substrate with a variant LovD polypeptide as defined above in the presence of an α-dimethylbutyryl thioester co-substrate and under conditions in which the monacolin J is converted to simvastatin.

[0029b] ~~Accordingly, in another aspect~~In one embodiment, the present disclosure provides methods of making simvastatin utilizing the variant LovD polypeptides described herein. The methods generally comprise contacting monacolin J with a variant LovD polypeptide in the presence of an α-dimethylbutyryl thioester co-substrate under conditions which yield simvastatin. In some embodiments, analogues of monacolin J may be used as precursor substrates to the formation of other statins, such as, but not limited to, huvastatin.

[0029c] In one aspect, there is provided a method of making simvastatin comprising contacting a lovastatin substrate with a variant LovD polypeptide as defined above in the presence of an α-dimethylbutyryl thioester co-substrate and under conditions in which the lovastatin substrate is converted to simvastatin.

[0030] The variant LovD polypeptides described herein recognize a number of different α-dimethylbutyryl thioester co-substrates, including by way of example and not limitation,

α-dimethylbutyryl-S-N-acetylcysteamine ("DMB-S-NAC"), α-dimethylbutyryl-S-methylthioglycolate ("DMB-S-MTG"), α-dimethylbutyryl-S-methyl mercaptopropionate "DMB-S-MMP"), α-dimethylbutyryl-S-ethyl mercaptoproprionate ("DMB-S-EMP"), α-dimethylbutyryl-S-methyl mercaptobutyrate ("DMB-S-MMB"), and α-dimethylbutyryl-S-merceaptopropionic acid ("DMB-S-MPA"). Any of these thioester co-substrates, or mixtures of such thioester co-substrates, can be used in the methods described herein.

[0031] In some embodiments, the reaction is carried out *in vitro* with an isolated variant LovD polypeptide, which can be purified or unpurified prior to use. In some embodiments, enzyme tags may be added to either terminus of the LovD polypeptide in order to enable binding to a

products. Scavenging includes, without limitation, chemical modification of thiol by-products, such as by oxidation. Suitable scavenging agents may include, but are not limited to, compounds that react with the thiol by-product and agents capable of chelating, adsorbing, absorbing or removing the thiol by-product. In some embodiments where a scavenging agent is used, the scavenging agent is activated charcoal. When used, the activated charcoal can be included in the reaction mixture in quantities ranging from 1 to 30 g/L, 2 to 20 g/L and 5 to 15 g/L.

[0035] The monacolin J substrate may be in the form of a salt, such as an ammonium or sodium salt. Reaction of the monacolin J substrate, such as the sodium or ammonium salts of monacolin J, may optionally be run in the presence of a scavenger, such as activated charcoal. Monacolin J may also be in the form of an ammonium salt. When the ammonium salt of monacolin J is converted to the ammonium salt of simvastatin, the ammonium salt of simvastatin may be precipitated from the reaction medium, for example, from water.

[0036] Other ~~aspects~~embodiments and advantages of the disclosure will be apparent from the detailed description that follows.

5. BRIEF DESCRIPTION OF THE FIGURES

[0037] FIG. 1 provides a diagram illustrating a pathway for synthesizing simvastatin from lovastatin or monacolin J;

[0038] FIG. 2 provides a polynucleotide sequence encoding wild type LovD enzyme from *Aspergillus terreus* that has been codon-optimized for expression in *E. coli.* (SEQ ID NO:1); and

[0039] FIG. 3 provides the polypeptide sequence encoded by the sequence of FIG. 2 (SEQ ID NO:2).

6. DETAILED DESCRIPTION

[0040] In certain aspects, the present disclosure provides LovD variant polypeptides that are capable of transferring an acyl group from certain thioester co-substrates to monacolin J and analogs or derivatives thereof to yield therapeutically important statin compounds, such as but not limited to simvastatin and huvastatin. The LovD variants have improved properties as compared to the wild-type LovD acyltransferase obtainable from *A. terreus* (SEQ ID NO:2), and can be used in cell-based or cell-free systems to efficiently and cost-effectively produce statins such as simvastatin from readily available starting materials, such as lovastatin and monacolin J.