elucidated (Nature 522 (2015), 497-501; Nature, 522 (2015), 502-505; Appl. Environ. Microbiol. 81 (2015), 4216- 4223).
Moreover, methods for the production of isobutene comprising the enzymatic conversion of 3-methylcrotonic acid into isobutene utilizing the above enzymes has previously been described wherein corresponding enzymes have artificially been implemented in a pathway which ultimately leads to the production of isobutene (WO 2017/085176). In WO 2017/085176 FMN-dependent decarboxylase enzymes catalysing the decarboxylation of 3-methylcrotonic acid into isobutene have been described which belong to the 3-polyprenyl-4-hydroxybenzoate decarboxylase (UbiD) family of enzymes while, *inter alia*, an UbiD enzyme derived from *Hypocrea atroviridis* (UniProt Accession number Q9NLP8) has been described.
Moreover, previously, improved variants of enzymes which are capable of converting 3-methylcrotonic acid into isobutene have been descried while the above FDC of *Hypocrea atroviridis* has been used as a model enzyme (WO 2017/191239).

Although the above means and methods allow to produce isobutene from 3-methylcrotonic acid while means and methods which allow to increase the production of isobutene from 3-methylcrotonic acid have already been described, there is still a need for further improvements, in particular as regards a further increase in efficiency of the process so as to make it more suitable for industrial purposes.

The present application addresses this need by providing the embodiments as defined in the claims.

SUMMARY

In one aspect, the present disclosure provides a variant of a 3-methylcrotonic acid decarboxylase (MDC) showing a higher activity in converting 3-methylcrotonic acid into isobutene over the MDC of SEQ ID NO:1,
wherein said variant is characterized in that:
it has an amino acid sequence having at least 80 % sequence identity to SEQ ID NO:1; and
it has one or more amino acid substitutions with another amino acid residue, deletions or insertions at a position selected from the group consisting of positions 406, 25, 35, 36, 74, 80, 86, 126, 129, 166, 196, 198, 204, 208, 236, 237, 238, 240, 298, 302, 359, 366, 368, 390, 395, 396, 402, 407, 408, 426, 449, 450, 451, 452, 454, 455, 456, 457, 458, 462 and 473 in the amino acid sequence shown in SEQ ID NO:1

or at a position corresponding to any of these positions.
In another aspect, the present disclosure provides a nucleic acid molecule encoding the MDC variant disclosed herein.
In yet another aspect, the present disclosure provides a vector comprising the nucleic acid molecule disclosed herein.
In one aspect, the present disclosure provides a host cell comprising the nucleic acid molecule disclosed herein or the vector disclosed herein.
In another aspect, the present disclosure provides use of the MDC variant disclosed herein or the host cell disclosed herein for the conversion of 3-methylcrotonic acid into isobutene.
In another aspect, the present disclosure provides a method for producing isobutene from 3-methylcrotonic acid by incubating 3-methylcrotonic acid with the MDC variant disclosed herein.
In yet another aspect, the present disclosure provides a composition comprising a variant of an MDC disclosed herein, the nucleic acid molecule disclosed herein, the vector disclosed herein or the host cell disclosed herein.
In one aspect, the present disclosure provides a composition comprising a variant of an MDC disclosed herein, the nucleic acid molecule disclosed herein, the vector disclosed herein or the host cell disclosed herein and 3-methylcrotonic acid.

DETAILED DESCRIPTION

In a first aspect, the present invention provides a variant of a 3-methylcrotonic acid decarboxylase (MDC) showing an improved activity in converting 3-methylcrotonic acid into isobutene over the corresponding MDC from which it is derived.
An improved enzyme variant or an enzyme variant capable of catalyzing a reaction with increased activity is defined as an enzyme variant which differs from the wildtype enzyme and which catalyzes the conversion of 3-methylcrotonic acid into isobutene so that the specific activity of the enzyme variant is higher than the specific activity of the wildtype enzyme for at least one given concentration of a 3-methylcrotonic acid (preferably any 3-methylcrotonic acid higher than 0 M and up to 1 M). A specific activity is defined as the number of moles of substrate converted to moles of product by unit of time by mole of enzyme. K_{cat} (turnover number) is the specific activity at saturating concentration of substrate.

CLAIMS

1. A variant of a 3-methylcrotonic acid decarboxylase (MDC) showing a higher activity in converting 3-methylcrotonic acid into isobutene over the MDC of SEQ ID NO:1,
wherein said variant is characterized in that:
it has an amino acid sequence having at least 80 % sequence identity to SEQ ID NO:1; and
it has one or more amino acid substitutions with another amino acid residue, deletions or insertions at a position selected from the group consisting of positions 406, 25, 35, 36, 74, 80, 86, 126, 129, 166, 196, 198, 204, 208, 236, 237, 238, 240, 298, 302, 359, 366, 368, 390, 395, 396, 402, 407, 408, 426, 449, 450, 451, 452, 454, 455, 456, 457, 458, 462 and 473 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions.

2. The MDC variant of claim 1, wherein
   (1) an amino acid residue at position 198 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glutamine or tryptophan; and/or
   (2) an amino acid residue at position 240 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine or asparagine; and/or
   (3) an amino acid residue at position 390 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with serine, aspartic acid, leucine, asparagine or glutamine; and/or
   (4) an amino acid residue at position 402 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine; and/or
   (5) an amino acid residue at position 406 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glycine, alanine or serine; and/or

(6) an amino acid residue at position 408 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine, alanine, cysteine, leucine, or methionine; and/or

(7) an amino acid residue at position 449 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine, methionine or valine; and/or

(8) an amino acid residue at position 25 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glutamic acid; and/or

(9) an amino acid residue at position 35 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine; and/or

(10) an amino acid residue at position 36 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with tyrosine; and/or

(11) an amino acid residue at position 74 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with valine; and/or

(12) an amino acid residue at position 80 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with serine; and/or

(13) an amino acid residue at position 86 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with asparagine or tyrosine; and/or

(14) an amino acid residue at position 126 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with valine; and/or

(15) an amino acid residue at position 129 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with valine; and/or

(16) an amino acid residue at position 166 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with aspartic acid; and/or

(17) an amino acid residue at position 196 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine; and/or

(18) an amino acid residue at position 204 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with arginine or serine; and/or

(19) an amino acid residue at position 208 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with leucine; and/or

(20) an amino acid residue at position 236 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with leucine or valine; and/or

(21) an amino acid residue at position 237 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, aspartic acid, asparagine or proline; and/or

(22) an amino acid residue at position 238 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with histidine, arginine, serine or tryptophan; and/or

(23) an amino acid residue at position 298 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with serine; and/or

(24) an amino acid residue at position 302 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, cysteine, phenylalanine, lysine, methionine, glutamine, arginine or serine; and/or

(25) an amino acid residue at position 359 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with cysteine or valine; and/or

(26) an amino acid residue at position 366 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine or phenylalanine; and/or

(27) an amino acid residue at position 368 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine or methionine; and/or

(28) an amino acid residue at position 395 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with cysteine, glutamic acid or valine; and/or

(29) an amino acid residue at position 396 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine; and/or

(30) an amino acid residue at position 407 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, cysteine, isoleucine, lysine, arginine, serine or threonine; and/or

(31) an amino acid residue at position 426 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with valine; and/or

(32) an amino acid residue at position 450 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, histidine, methionine, asparagine, glutamine, serine or valine; and/or

(33) an amino acid residue at position 451 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with asparagine or serine; and/or

(34) an amino acid residue at position 452 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with phenylalanine; and/or

(35) an amino acid residue at position 454 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, aspartic acid, glycine, histidine, lysine, leucine, methionine, glutamine, arginine, serine, threonine, valine or tryptophan; and/or

(36) an amino acid residue at position 455 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, glutamic acid, histidine, lysine, asparagine, serine or valine; and/or

(37) an amino acid residue at position 456 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glutamine; and/or

(38) an amino acid residue at position 457 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with lysine; and/or

(39) an amino acid residue at position 458 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glutamic acid, phenylalanine, glycine,

leucine, methionine, asparagine, glutamine, arginine, serine or tyrosine; and/or

(40) an amino acid residue at position 462 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with cysteine or valine; and/or

(41) an amino acid residue at position 473 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with serine.

3. The MDC variant of claim 1 or 2, wherein said variant is characterized in that it has one or more further amino acid substitutions, deletions or insertions at a position selected from the group consisting of 2, 3, 5, 6, 24, 64, 66, 85, 154, 168, 175, 195, 197, 214, 241, 277, 296, 305, 339, 343, 347, 349, 350, 363, 391, 394, 398, 401, 403, 404, 405, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 453, 459, 460, 461, 479 and 496 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions, and wherein said MDC has an improved activity in converting 3-methylcrotonic acid into isobutene over the corresponding MDC from which it is derived.

4. The MDC variant of claim 3, wherein

(42) an amino acid residue at position 85 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with leucine; and/or

(43) an amino acid residue at position 241 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with aspartic acid, asparagine or valine; and/or

(44) an amino acid residue at position 401 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with tyrosine; and/or

(45) an amino acid residue at position 403 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, cysteine, glycine, histidine, isoleucine, asparagine, proline, glutamine, arginine or valine; and/or

(46) an amino acid residue at position 404 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with phenylalanine, leucine or methionine; and/or

(47) an amino acid residue at position 405 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is

deleted or substituted with histidine, isoleucine, methionine, threonine or valine; and/or

(48) an amino acid residue at position 443 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, aspartic acid, phenylalanine, histidine, leucine, asparagine, arginine, serine, tryptophan or tyrosine; and/or

(49) an amino acid residue at position 444 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, glutamic acid, phenylalanine, histidine, isoleucine, leucine, glutamine or threonine; and/or

(50) an amino acid residue at position 445 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with leucine, alanine, cysteine, serine or threonine; and/or

(51) an amino acid residue at position 446 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, cysteine, phenylalanine, isoleucine, methionine, asparagine, serine or valine; and/or

(52) an amino acid residue at position 448 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with phenylalanine, tryptophan or tyrosine; and/or

(53) an amino acid residue at position 2 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with asparagine; and/or

(54) an amino acid residue at position 3 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with leucine or methionine; and/or

(55) an amino acid residue at position 5 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine; and/or

(56) an amino acid residue at position 6 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with methionine; and/or

(57) an amino acid residue at position 24 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, aspartic acid, leucine or valine; and/or

(58) an amino acid residue at position 64 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with histidine; and/or

(59) an amino acid residue at position 66 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with histidine; and/or

(60) an amino acid residue at position 154 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with aspartic acid; and/or

(61) an amino acid residue at position 168 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with valine; and/or

(62) an amino acid residue at position 175 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glutamic acid; and/or

(63) an amino acid residue at position 195 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine or valine; and/or

(64) an amino acid residue at position 197 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with valine; and/or

(65) an amino acid residue at position 214 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with threonine; and/or

(66) an amino acid residue at position 277 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with tyrosine; and/or

(67) an amino acid residue at position 296 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glutamine; and/or

(68) an amino acid residue at position 305 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with isoleucine or threonine; and/or

(69) an amino acid residue at position 339 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with histidine; and/or

(70) an amino acid residue at position 343 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with serine; and/or

(70) an amino acid residue at position 347 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with cysteine; and/or

(72) an amino acid residue at position 349 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with methionine or serine; and/or

(73) an amino acid residue at position 350 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with serine; and/or

(74) an amino acid residue at position 363 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glycine; and/or

(75) an amino acid residue at position 391 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with aspartic acid or tryptophan; and/or

(76) an amino acid residue at position 394 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with cysteine, aspartic acid, glutamine or threonine; and/or

(77) an amino acid residue at position 398 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with cysteine or arginine; and/or

(78) an amino acid residue at position 439 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with cysteine; and/or

(79) an amino acid residue at position 440 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with leucine; and/or

(80) an amino acid residue at position 441 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glycine, histidine, lysine, asparagine, proline, arginine, serine or threonine; and/or

(81) an amino acid residue at position 442 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, cysteine, glutamic acid, glycine,

histidine, leucine, methionine, asparagine, glutamine, arginine, serine or threonine; and/or

(82) an amino acid residue at position 447 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glutamine; and/or

(83) an amino acid residue at position 453 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with cysteine, glycine, leucine, asparagine, proline, arginine or serine; and/or

(84) an amino acid residue at position 459 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with aspartic acid, glutamic acid, glycine, methionine, asparagine, glutamine, arginine, threonine or tyrosine; and/or

(85) an amino acid residue at position 460 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with glutamic acid, phenylalanine, glycine, leucine, methionine, asparagine, glutamine or arginine; and/or

(86) an amino acid residue at position 461 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with alanine, glycine, leucine, methionine, glutamine, serine or valine; and/or

(87) an amino acid residue at position 479 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with cysteine, isoleucine, leucine, methionine, serine, threonine or valine; and/or

(88) an amino acid residue at position 496 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position, is deleted or substituted with leucine.

5. A nucleic acid molecule encoding the MDC variant of any one of claims 1 to 4.

6. A vector comprising the nucleic acid molecule of claim 5.

7. A host cell comprising the nucleic acid molecule of claim 5 or the vector of claim 6.

8. Use of the MDC variant of any one of claims 1 to 4 or the host cell of claim 7 for the conversion of 3-methylcrotonic acid into isobutene.

9. A method for producing isobutene from 3-methylcrotonic acid by incubating 3-methylcrotonic acid with the MDC variant of any one of claims 1 to 4.

10. The method of claim 9, wherein the enzymatic conversion is carried out in vitro.

11. A composition comprising a variant of an MDC of any one of claims 1 to 4, the nucleic acid molecule of claim 5, the vector of claim 6 or the host cell of claim 7.

12. A composition comprising a variant of an MDC of any one of claims 1 to 4, the nucleic acid molecule of claim 5, the vector of claim 6 or the host cell of claim 7 and 3-methylcrotonic acid.