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DESCRIPTION

[0001] The present invention relates to mevalonate diphosphate decarboxylase variants having improved activity in converting 3-phosphonoxyisovalerate into isobutene. Moreover, the present invention relates to enzymes which are characterized in that they are capable of converting 3-phosphonoxyisovalerate into isobutene with a k_{cat} of more than 0.1 s^{-1} .

[0002] A large number of chemical compounds are currently derived from petrochemicals. Alkenes (such as ethylene, propylene, the different butenes, or else the pentenes, for example) are used in the plastics industry, for example for producing polypropylene or polyethylene, and in other areas of the chemical industry and that of fuels. Over the past years, the bioproduction of plastics ("bioplastics") and biofuels has become a thriving field due to economic concerns linked to the price of oil, and to environmental considerations that are both global (carbon-neutral products) and local (waste management). Thus, there is a need for efficient enzymes for producing alkenes such as isobutene.

[0003] WO 2010/001078 describes a process for producing alkenes, such as isobutene, by enzymatic conversion of 3-hydroxyalkanoic acids with an enzyme having the activity of a decarboxylase, for example a mevalonate diphosphate (MDP) decarboxylase. Such a method is advantageous because it helps to avoid the use of petroleum products, to lower the costs of producing plastics and fuels and can have a considerable global environmental impact by allowing carbon to be stored in solid form. It could be shown that mevalonate diphosphate decarboxylase is capable of using substrates other than its natural substrate mevalonate diphosphate, in particular 3-hydroxyalkanoic acids, and convert them into terminal alkenes. Mevalonate diphosphate (MDP) decarboxylase (enzyme nomenclature EC 4.1.1.33) is an enzyme involved in cholesterol biosynthesis. The enzyme has been isolated from a variety of organisms including animals, fungi, yeasts and some bacteria. It can also be expressed by some plants (Lalitha et al., *Phytochemistry* 24 (11), (1985), 2569-2571). Many genes encoding this enzyme have been cloned and sequenced. These enzymes are generally composed of 300 to 400 amino acids and use ATP as co-substrate, which is converted during the reaction to ADP and inorganic phosphate. The phosphate group is transferred in a first step from the ATP molecule to the tertiary alcohol of mevalonate diphosphate, releasing ADP. The reaction intermediate, which is phosphorylated on the 3-hydroxyl group, undergoes in a second step elimination of the phosphate group and decarboxylation, in the physiological case releasing isopentenyl diphosphate.

MDP decarboxylases have been isolated from a multitude of different organisms, eukaryotic and prokaryotic, and have been analyzed and characterized in detail. Also, various mutants have been produced in order to identify the amino acid residues which may play a crucial role in the enzymatic activity of the enzyme. For example, Alvear et al. (*Biochemistry* 21 (1982), 4646-4650) describe the purification and characterization of avian liver MDP decarboxylase and Dhe-Paganon et al. (*Biochemistry* 33 (1994), 13355-13362) describe the mechanism of the reaction catalyzed by MDP decarboxylase. Berges et al. (*J. Bacteriol.* 179 (1997), 4664-4670) reported on a mutation of the *S. cerevisiae* MDP decarboxylase which leads to thermosensitivity. Krepiy and Miziorko (*Protein Sci.* 13 (2004), 1875-1881) identified active site residues in MDP decarboxylase of yeast and analysed mutations which led to a reduction in activity. Similarly, Krepiy and Miziorko (*Biochemistry* 44 (2005), 2671-2677) investigated conserved serine residues which are located in a proposed interdomain active site cleft of MDP decarboxylase as to their relevance and could show that mutation of any of these serine residues leads to a reduction or loss of activity. Also Qiu et al. (*Bioorganic & medicinal Chemistry Letters* 17 (2007), 6164-6168) analyze MDP decarboxylase (of rat) and report on various mutants which lead to a reduction or loss of activity. Voynova et al. (*Arch. Biochem. Biophys.* 480 (2008), 58-67) characterized the human MDP decarboxylase and identified several amino acid residues which lead to a decrease or loss of enzyme activity.

Moreover, the crystal structure of several MDP decarboxylases and MDP decarboxylase mutants from different origins has been established, e.g. for the *Staphylococcus epidermidis* enzyme (Barta et al., *J. Biol. Chem.* 286 (2011), 23900-23910; Barta et al., *Biochemistry* 51 (2012), 5611-5621; PDB accession numbers 3QT5-6-7-8 and 4DPX, 4DPY, 4DPU, 4DPT, 4DU8, 4DU7 and 4DPW), for the *Trypanosoma brucei* and *Staphylococcus aureus* enzymes (Byres et al. *J. Mol. Biol.* 371 (2007), 540-553; PDB accession number 2HKE, 2HK2, 2HK3), the human

enzyme (Voynova et al. (Arch. Biochem. Biophys. 480 (2008), 58-67; PDB accession number 3D4J), the *Streptococcus pyogenes* enzyme (PDB accession number 2GS8), the mouse enzyme (PDB accession number 3F0N), the *Legionella pneumophila* enzyme (PDB accession number 3LTO) and the *S. cerevisiae* enzyme (Bonanno et al., Proc. Natl. Acad. Sci. USA. 98 (2001), 12896-12901; PDB accession number 1FI4).

Lefurgy et al. (J. Biol. Chem. 285 (2010), 20654-20663) analyzed the ligand binding pocket of MDP decarboxylase of *Streptococcus pneumoniae* by using a series of MDP analogues and Weerasinghe and Dassanayake (J. Mol. Model. 16 (2010) 489-498) reported on the simulation of structural and functional properties of MDP decarboxylase of *S. cerevisiae* using the wild-type enzyme and the thermosensitive mutant described in Berges et al. (J. Bacteriol. 179 (1997), 4664-4670).

Byres et al. (J. Mol. Biol. 371 (2007), 540-553) compared the crystal structures of different MDP decarboxylases, in particular of those from *T. brucei*, *S. aureus* and *S. cerevisiae* and observe that the overall architecture of the enzyme remains conserved.

WO 2010/001078 discloses inter alia that it is possible to convert 3-hydroxy-3-methylbutyrate (or 3-hydroxyisovalerate) into isobutene by a decarboxylase, in particular an MDP decarboxylase. In this case, the reaction intermediate is 3-phosphonoxyisovalerate which is further converted in the second part of the reaction into isobutene. Gogerty et al. (Appl. Environ. Microbiol. 76 (2010), 8004-8010) also report on the formation of isobutene from 3-hydroxy-3-methylbutyrate using an MDP decarboxylase from *S. cerevisiae* and show that mutations at residues 145 and 74 of this enzyme, which are located within or close to the proposed active site of this enzyme, lead to an increase of the conversion of 3-hydroxy-3-methylbutyrate into isobutene. However, the level of production of isobutene achieved is still too low for commercial application.

Later works have shown that different MDP decarboxylases may show different efficiencies as regards the catalysis of the first and the second step of the reaction as described above, with some MDP decarboxylases showing a high activity in the first step and others showing a high activity in the second step. Therefore, it had been proposed to combine two MDP decarboxylases which show a high activity in the first and in the second step of the reaction, respectively, so as to optimize the overall enzymatic reaction (WO2012/052427).

However, although such a method allows to produce alkenes by enzymatically converting 3-hydroxyalkanoates (e.g. isobutene from 3-hydroxyisovalerate), there is still a need for improvements, in particular as regards a further increase in efficiency of the process so as to make it more suitable for industrial purposes.

[0004] The present invention relates to the embodiments as characterized in the claims. Thus, it relates to the following items.

1. 1. A variant of a mevalonate diphosphate decarboxylase showing an improved activity in converting 3-phosphonoxyisovalerate into isobutene over the mevalonate diphosphate decarboxylase of SEQ ID NO:1, wherein the mevalonate diphosphate decarboxylase variant is characterized in that it has an amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 60% sequence identity to SEQ ID NO:1, in which the amino acid residue at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to said position in said sequence having at least 60 % sequence identity to SEQ ID NO:1, is substituted with another amino acid residue or.
2. 2. The mevalante diphosphate decarboxylase variant of item 1, wherein:
the amino acid residue at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in said sequence having at least 60% sequence identity to SEQ ID NO:1, is deleted or substituted with cysteine, serine, glutamic acid, glycine, glutamine, threonine, valine, alanine or aspartic acid.
3. 3. The mevalonate diphosphate decarboxylase variant of item 1 or 2, wherein at least one further amino acid residue is substituted or deleted at a position selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315 in SEQ ID NO:1 or at a position corresponding to this position in said sequence having at least 60% sequence identity to SEQ ID NO:1.
4. 4. The mevalonate diphosphate decarboxylase variant of item 3, wherein the amino acid residues at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this

position in said sequence having at least 60% sequence identity to SEQ ID NO:1 is deleted or substituted with another amino acid residue and wherein the amino acid residues at position 121 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in said sequence having at least 60% sequence identity to SEQ ID NO:1 is deleted or substituted with another amino acid residue.

5. 5. The mevalonate diphosphate decarboxylase variant of item 3, wherein:

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

(2) 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 in a related sequence, is deleted or substituted with histidine; and/or

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

(4) an amino acid residue at position 11 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine, glutamic acid or phenylalanine, preferably cysteine; and/or

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

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

(7) 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 in a related sequence, is deleted or substituted with arginine, serine, or leucine; and/or

(8) an amino acid residue at position 28 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with lysine or alanine; and/or

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

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

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

(12) an amino acid residue at position 45 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine, phenylalanine, methionine or valine, preferably leucine; and/or

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

(14) an amino acid residue at position 57 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with serine; and/or

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

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

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

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

(19) 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 in a related sequence, is deleted or substituted with glycine; and/or

(20) 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 in a related sequence, is deleted or substituted with glutamine; and/or

(21) an amino acid residue at position 87 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or

(22) an amino acid residue at position 91 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with histidine; and/or

(23) an amino acid residue at position 105 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine; and/or

(24) an amino acid residue at position 111 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with methionine; and/or

(25) an amino acid residue at position 116 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with arginine, isoleucine, leucine, serine or methionine, preferably arginine or isoleucine; and/or

(26) an amino acid residue at position 118 in the amino acid sequence shown in SEQ ID NO:1 at a position corresponding to this position in a related sequence, is deleted or substituted with leucine or tryptophane; and/or

(27) an amino acid residue at position 120 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with asparagine, leucine, arginine, isoleucine or valine, preferably asparagine, leucine, arginine or isoleucine; and/or

(28) an amino acid residue at position 121 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with arginine, leucine, tryptophan, phenylalanine, tyrosine, asparagine or lysine, preferably arginine or phenylalanine; and/or

(29) the amino acid residue at position 122 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is substituted with methionine or tyrosine; and/or

(30) an amino acid residue at position 123 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with methionine or arginine; and/or

(30) 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 in a related sequence, is deleted or substituted with proline or valine; and/or

(32) an amino acid residue at position 134 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glycine; and/or

(33) an amino acid residue at position 139 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine or alanine; and/or

(34) an amino acid residue at position 141 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with proline, cysteine, glycine or threonine; and/or

(35) an amino acid residue at position 142 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine; and/or

(36) an amino acid residue at position 159 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine; and/or

(37) an amino acid residue at position 160 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with valine; and/or

(38) an amino acid residue at position 161 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with arginine; and/or

(39) an amino acid residue at position 164 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamine; and/or

(40) 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 in a related sequence, is deleted or substituted with serine; and/or

(41) an amino acid residue at position 173 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine; and/or

(42) an amino acid residue at position 177 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with valine or cysteine, preferably valine; and/or

(43) an amino acid residue at position 179 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with lysine or leucine; and/or

(44) an amino acid residue at position 180 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with proline; and/or

(45) an amino acid residue at position 182 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or

(46) an amino acid residue at position 186 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with histidine, leucine, valine, isoleucine or asparagine; and/or

(47) an amino acid residue at position 188 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine; and/or

(48) 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 in a related sequence, is deleted or substituted with aspartic acid; and/or

(49) 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 in a related sequence, is deleted or substituted with leucine; and/or

(50) an amino acid residue at position 205 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with histidine; and/or

(51) 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 in a related sequence, is deleted or substituted with leucine; and/or

(52) an amino acid residue at position 215 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine; and/or

(53) an amino acid residue at position 221 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or

(54) an amino acid residue at position 227 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with lysine; and/or

(55) an amino acid residue at position 231 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamine or leucine; and/or

(56) 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 in a related sequence, is deleted or substituted with arginine, glutamic acid or lysine; and/or

(57) 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 in a related sequence, is deleted or substituted with methionine or isoleucine; and/or

(58) an amino acid residue at position 242 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine or glutamic acid; and/or

(59) an amino acid residue at position 246 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or

(60) an amino acid residue at position 248 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with threonine; and/or

(61) an amino acid residue at position 251 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with methionine, phenylalanine or valine, preferably methionine; and/or

(62) an amino acid residue at position 252 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or

(63) an amino acid residue at position 253 in the amino acid sequence shown in SEQ ID NO:1 or at a

position corresponding to this position in a related sequence, is deleted or substituted with valine or isoleucine; and/or

(64) an amino acid residue at position 255 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or

(65) an amino acid residue at position 258 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine; and/or

(66) an amino acid residue at position 264 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamine; and/or

(67) an amino acid residue at position. 267 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with arginine; and/or

(68) an amino acid residue at position 279 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine; and/or

(69) an amino acid residue at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine, serine, glutamic acid, glycine, glutamine, threonine, valine, alanine or aspartic acid; and/or

(70) an amino acid residue at position 291 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with aspartic acid; and/or

(71) an amino acid residue at position 293 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with phenylalanine or tryptophan; and/or

(72) an amino acid residue at position 297 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine or leucine, preferably cysteine; and/or

(73) an amino acid residue at position 299 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with proline or lysine; and/or

(74) an amino acid residue at position 303 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with methionine; and/or

(75) an amino acid residue at position 307 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with histidine; and/or

(76) an amino acid residue at position 308 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with serine; and/or

(77) an amino acid residue at position 315 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with serine.

6. 6. The mevalonate diphosphate decarboxylase variant of any one of items 1 to 5 which has an amino acid sequence as shown in SEQ ID NO: 1 wherein the following substitutions have been effected:

1. (i) K24R-C118L-Y121R-E159L-M173C-E177C-K282C-E291 D-F297L-L303M-T308S; or
2. (ii) Y121R-E159L-M173C-K282C-L303M-T308S.

7. 7. The mevalonate diphosphate decarboxylase variant of item 6 (i), which shows the following additional substitutions:

S141P-I16L-K241I-S248T-M28K-K180P; or

S141P-I16L-R91H-K241M-S248T-Q299K.
8. 8. A nucleic acid molecule encoding the mevalonate diphosphate decarboxylase variant of any one of items 1 to 7.
9. 9. A vector comprising the nucleic acid molecule of item 8.
10. 10. A host cell comprising the vector of item 9.
11. 11. A method for providing a variant of a mevalonate diphosphate decarboxylase wherein said variant shows an improved activity of converting 3-phosphonoxyisovalerate into isobutene over the mevalonate diphosphate decarboxylase of SEQ ID NO:1, said method comprising the step of effecting a change in the sequence of the mevalonate diphosphate decarboxylase wherein said change is effected at the amino acid position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any one of these positions in a sequence having at least 60% sequence identity to SEQ ID NO: 1, wherein said change is a substitution or deletion.
12. 12. The method of item 11, wherein said method further comprises the step of effecting one or more further changes in the sequence of the mevalonate diphosphate decarboxylase wherein said change(s) is/are effected at one or more amino acid positions selected from the group consisting of the amino acid positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any one of these positions in said sequence having at least 60% sequence identity to SEQ ID NO:1.
13. 13. The method of item 11 or 12 wherein the changes which are effected in a mevalonate diphosphate decarboxylase at one or more positions corresponding to positions corresponding to positions 282, 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315 in the amino acid sequence shown in SEQ ID NO:1 are selected from the group consisting of those identified in items (1) to (77) of item 6.
14. 14. Use of the mevalonate diphosphate decarboxylase of any one of items 1 to 7 or the host cell of item 10 for
 1. (i) the conversion of 3-hydroxyisovalerate or of 3-phosphonoxyisovalerate into isobutene;
 2. (ii) the conversion of mevalonate or of mevalonate-3-phosphate into isoprenol; or
 3. (iii) the conversion of 3-hydroxypent-4-enoate or of 3-phosphonoxypent-4-enoate into 1,3-butadiene.
15. 15. A method for producing isobutene from 3-hydroxyisovalerate or from 3-phosphonoxyisovalerate comprising the steps of:
 1. (i) culturing the host cell of item 10 in a suitable medium; and
 2. (ii) recovering the produced isobutene.
16. 16. A method for producing isoprenol from mevalonate or from mevalonate-3-phosphate comprising the steps of:
 1. (i) culturing the host cell of item 10 in a suitable medium; and
 2. (ii) recovering the produced isoprenol.
17. 17. A method for producing 1,3-butadiene from 3-hydroxypent-4-enoate or from 3-phosphonoxypent-4-enoate comprising the steps of:
 1. (i) culturing the host cell of item 10 in a suitable medium; and
 2. (ii) recovering the produced 1,3-butadiene.

[0005] In particular, there are disclosed enzymes which are characterized in that they are capable of converting 3-phosphonoxyisovalerate into isobutene with a k_{cat} of more than 0.01 s^{-1} or 0.1 s^{-1} , preferably of more than 1 s^{-1} , more preferably of more than 10 s^{-1} , and even more preferably of more than 10^2 s^{-1} or most preferably of more than 10^3 s^{-1} . Preferably such enzymes are variants of a mevalonate diphosphate decarboxylase, and even more preferably such enzymes have an amino acid sequence which shows more than 60% sequence homology to the amino acid sequence shown in SEQ ID NO:1. It is preferred that the enzymes according to the invention are non-naturally occurring enzymes. This means that they substantially differ from naturally occurring enzymes, in particular as regards their primary structure, i.e., the amino acid sequence. Thus, they show an amino acid sequence which does not occur in nature. It is preferable that such non-naturally occurring enzymes also differ from naturally occurring enzymes insofar that they have a higher enzymatic activity for the reactions described herein.

[0006] Thus, the present invention provides in particular variants of mevalonate diphosphate decarboxylase which show an improved activity as regards the conversion of 3-phosphonoxyisovalerate into isobutene thereby allowing to dramatically increase the production efficiency of isobutene from 3-hydroxyisovalerate.

The term "mevalonate diphosphate decarboxylase" in the context of the present invention refers to an enzyme which naturally has the ability to convert mevalonate diphosphate into isopentenyl diphosphate and which is classified as EC 4.1.1.33. The term "mevalonate diphosphate decarboxylase" also covers enzymes which are classified as a mevalonate diphosphate decarboxylase and which act on mevalonate monophosphate, i.e. which are mevalonate monophosphate decarboxylases. An example for such an enzyme is the enzyme from *Roseiflexus* sp. (strain RS-1) (Uniprot Accession number: A5V173) which is classified as a mevalonate diphosphate decarboxylase. Since some bacteria from *Roseiflexus* genus, e.g. *Roseiflexus castenholzii*, have been reported to have an alternative mevalonate pathway which involves the action of a mevalonate monophosphate decarboxylase an enzyme from *Roseiflexus* sp. may act as a mevalonate monophosphate decarboxylase. Additionally, a third mevalonate pathway has recently been described in *T. acidophilum*. In this pathway, the formation of isopentenyl monophosphate probably occurs through mevalonate-3,5-pyrophosphate (MVA-3,5-PP). The two genes which can be implicated in the decarboxylation of this intermediate are Ta0461 and Ta0893. These genes can be associated to the family of diphosphomevalonate decarboxylase referenced in InterPro database as [IPR005935](http://www.ebi.ac.uk/interpro/entry/IPR005935) (<http://www.ebi.ac.uk/interpro/entry/IPR005935>), but these two enzymes may act as mevalonate-3,5-pyrophosphate decarboxylase. Thus, the term "mevalonate diphosphate decarboxylase" also covers such enzymes. The present invention relates to variants which are derived from a mevalonate diphosphate decarboxylase. The variants of such an enzyme according to the present invention are characterized by the feature that they are derived from an MDP decarboxylase having the amino acid sequence shown in SEQ ID NO:1 or having a related sequence

at least 60% or at least 90% identical) and in which mutations are effected at one or more of the positions as indicated herein below and by the feature that they show the ability to convert 3-phosphonoxyisovalerate into isobutene and that they can do this with an improved activity. In a preferred embodiment the variant according to the present invention is derived from a sequence which shows at least 80% sequence identity to SEQ ID NO:1 and in which one or more substitutions and/or deletions and/or insertions at the positions indicated herein below have been effected.

[0007] Given the high conservation between MDP decarboxylases, the teaching of the present invention is not restricted to the enzyme of *S. mitis* (represented by SEQ ID NO: 1) but can be extended to MDP decarboxylases from other organisms. Thus, the present invention also relates to variants of MDP decarboxylase which are derived from enzymes which are structurally related to the *S. mitis* sequence and which show one or more substitutions and/or deletions and/or insertions at positions corresponding to any of the positions as indicated herein-below. The term "structurally related" refers to MDP decarboxylases which show a sequence identity of at least $n\%$ to the sequence shown in SEQ ID NO: 1 with n being an integer between 40 and 100, preferably 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99. In a preferred embodiment the structurally related MDP decarboxylase is of prokaryotic origin. These variants are characterized by the feature

that they show the ability to convert 3-phosphonoxyisovalerate into isobutene and that they can do this with an improved activity when compared to the enzyme from which these variants are derived. Even more preferably such variants also show an improved activity when compared to the enzyme having the amino acid sequence shown in SEQ ID NO:1.

Thus, the variant of MDP decarboxylase has or preferably is derived from a sequence which is at least n % identical to SEQ ID NO:1 with n being an integer between 60 and 100, preferably

65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99, and it has (a) substitution(s) and/or (a) deletion(s) and/or (an) insertion(s) at a position as indicated below. When the sequences which are compared do not have the same length, the degree of identity either refers to the percentage of amino acid residues in the shorter sequence which are identical to amino acid residues in the longer sequence or to the percentage of amino acid residues in the longer sequence which are identical to amino acid residues in the shorter sequence. Preferably, it refers to the percentage of amino acid residues in the shorter sequence which are identical to amino acid residues in the longer sequence. The degree of sequence identity can be determined according to methods well known in the art using preferably suitable computer algorithms such as CLUSTAL.

When using the Clustal analysis method to determine whether a particular sequence is, for instance, at least 40%, 50% or 60% or 90% identical to a reference sequence default settings may be used or the settings are preferably as follows: Matrix: blosum 30; Open gap penalty: 10.0; Extend gap penalty: 0.05; Delay divergent: 40; Gap separation distance: 8 for comparisons of amino acid sequences. For nucleotide sequence comparisons, the Extend gap penalty is preferably set to 5.0.

In a preferred embodiment ClustalW2 is used for the comparison of amino acid sequences. In the case of pairwise comparisons/alignments, the following settings are preferably chosen: Protein weight matrix: BLOSUM 62; gap open: 10; gap extension: 0.1. In the case of multiple comparisons/alignments, the following settings are preferably chosen: Protein weight matrix: BLOSUM 62; gap open: 10; gap extension: 0.2; gap distance: 5; no end gap.

Preferably, the degree of identity is calculated over the complete length of the sequence.

[0008] Examples for MDP decarboxylases which show a sequence identity of at least 60%, in particular between 60% and 80%, to SEQ ID NO:1 are shown in the following Table.

Table 1

Entry name UniprotKb	Accession	Organism	Entry date	Last Database update	Entry version	Sequence version	% identity with SEQ ID NO:1
F5U3H6_STRAP	F5U3H6	Streptococcus anginosus SK52	July 27, 2011	October 19,2011	3	1	77
A8AUU9_STRGC	A8AUU9	Streptococcus gordonii (strain Challis / ATCC 35105 / CH1 / DL1 / V288)	October 23,2011	October 19,2011	29	1	74
F2C8L7_STRSA	F2C8L7	Streptococcus sanguinis SK330	May 31, 2011	October 19,2011	4	1	74
E7S990_9STRE	E7S990	Streptococcus australis ATCC 700641	April 5,2011	October 19,2011	4	1	71
E7S3Q3_STRAG	E7S3Q3	Streptococcus agalactiae ATCC 13813	April 5,2011	October 19,2011	4	1	71
F0VY81_STRG2	F0VY81	Streptococcus	May 3,	October	5	1	71

Entry name UniprotKb	Accession	Organism	Entry date	Last Database update	Entry version	Sequence version	% identity with SEQ ID NO:1
		gallolyticus (strain ATCC BAA-2069)	2011	19,2011			
B9DU65_STRU0	F0VY81	Streptococcus uberis (strain ATCC BAA- 854 / 0140J)	March 24, 2009	October 19,2011	21	1	70
E8JNQ3_STREI	E8JNQ3	Streptococcus equinus ATCC 9812	April 5, 2011	October 19,2011	4	1	70
E0PWI4_STRPY	E0PWI4	Streptococcus pyogenes ATCC 10782	November 2, 2010	October 19,2011	7	1	70
Q9FD58_STRPY	Q9FD58	Streptococcus pyogenes	March 27, 2001	October 19,2011	49	1	70
D0GIF6_9FUSO	D0GIF6	Leptotrichia goodfellowii F0264	December 15, 2009	October 19,2011	19	1	62

[0009] Examples for MDP decarboxylases which show a sequence identity of at least 80%, in particular between 80% and 90%, to SEQ ID NO:1 are shown in the following Table.

Table 2

Entry name UniprotKb	Accession	Organism	Entry date	Last update	Entry version	Sequence version	% identity with SEQ ID NO:1
E8KA62_9STRE	E8KA62	Streptococcus peroris ATCC700780	April 5, 2011	October 19,2011	5	1	89
E8JYA8_9STRE	E8JYA8	Streptococcus infantis ATCC700779	April 5, 2011	October 19,2011	4	1	88
F5VZT1_9STRE	F5VZT1	Streptococcus infantis SK1076	July 27, 2011	October 19,2011	3	1	88

[0010] Examples for MDP decarboxylases which show a sequence identity of at least 90%, in particular between 90% and 100%, to SEQ ID NO:1 are shown in the following Table.

Table 3

Entry name UniprotKb	Accession	Organism	Entry date	Last update	Entry version	Sequence version	% identity with SEQ ID NO:1
E1LJ67_STRMT	E1LJG7	Streptococcus mitis SK321	November 30, 2010	October 19, 2011	8	1	96

Entry name UniprotKb	Accession	Organism	Entry date	Last update	Entry version	Sequence version	% identity with SEQ ID NO:1
E9FM52_9STRE	E9FM52	Streptococcus sp M334	April 5, 2011	October 19, 2011	5	1	95
E6KKU6_STRSA	E6KKU6	Streptococcus sanguinis ATCC 49296	March 8, 2011	October 19, 2011	4	1	95
D4FR73-STROR	D4FR73	Streptococcus oralis ATCC 35037	May 18, 2010	October 19, 2011	9	1	94
Q8DR50_STRR6	Q8DR50	Streptococcus pneumonia ATCC BAA- 255/R6	March 1, 2003	October 19, 2011	58	1	94

[0011] Amino acid residues located at a position corresponding to a position as indicated herein-below in the amino acid sequence shown in SEQ ID NO:1 can be identified by the skilled person by methods known in the art. For example, such amino acid residues can be identified by aligning the sequence in question with the sequence shown in SEQ ID NO:1 and by identifying the positions which correspond to the above indicated positions of SEQ ID NO:1. The alignment can be done with means and methods known to the skilled person, e.g. by using a known computer algorithm such as the Lipman-Pearson method (Science 227 (1985), 1435) or the CLUSTAL algorithm. It is preferred that in such an alignment maximum homology is assigned to conserved amino acid residues present in the amino acid sequences.

In a preferred embodiment ClustalW2 is used for the comparison of amino acid sequences. In the case of pairwise comparisons/alignments, the following settings are preferably chosen: Protein weight matrix: BLOSUM 62; gap open: 10; gap extension: 0.1. In the case of multiple comparisons/alignments, the following settings are preferably chosen: Protein weight matrix: BLOSUM 62; gap open: 10; gap extension: 0.2; gap distance: 5; no end gap.

When the amino acid sequences of MDP decarboxylases are aligned by means of such a method, regardless of insertions or deletions that occur in the amino acid sequences, the positions of the corresponding amino acid residues can be determined in each of the MDP decarboxylases. Examples of alignments are provided in Figures 10 to 12.

[0012] In the context of the present invention, "substituted with another amino acid residue" means that the respective amino acid residues at the indicated position can be substituted with any other possible amino acid residues, e.g. naturally occurring amino acids or non-naturally occurring amino acids (Brustad and Arnold, Curr. Opin. Chem. Biol. 15 (2011), 201-210), preferably with an amino acid residue selected from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. Preferred substitutions for certain positions are indicated further below. Moreover, the term "substituted" or "substitution" also means that the respective amino acid residue at the indicated position is modified.

[0013] Such modifications include naturally occurring modifications and non-naturally occurring modifications. Naturally occurring modifications include but are not limited to eukaryotic post-translational modification, such as attachment of functional groups (e.g. acetate, phosphate, hydroxyl, lipids (myristoylation of glycine residues) and carbohydrates (e.g. glycosylation of arginine, asparagines etc.). Naturally occurring modifications also encompass the change in the chemical structure by citrullination, carbamylation and disulphide bond formation between cysteine residues; attachment of co-factors (FMN or FAD that can be covalently attached) or the

attachement of peptides (e.g. ubiquitination or sumoylation).

Non-naturally occurring modifications include, e.g., in vitro modifications such as biotinylation of lysine residue or the inclusion of non-canonical amino acids (see Liu and Schultz, *Annu. Rev. Biochem.* 79 (2010), 413-44 and Wang et al., *Chem. Bio.* 2009 March 27; 16 (3), 323-336; doi:101016/jchembiol.2009.03.001).

[0014] In the context of the present invention, "deleted" or "deletion" means that the amino acid at the corresponding position is deleted.

In the context of the present invention, "inserted" or "insertion" means that at the respective position one or two, preferably one amino acid residue is inserted, preferable in front of the indicated position.

[0015] Thus, there is disclosed a variant of a mevalonate diphosphate decarboxylase showing an improved activity in converting 3-phosphonoxyisovalerate into isobutene over the corresponding mevalonate diphosphate decarboxylase from which it is derived, wherein the mevalonate diphosphate decarboxylase variant is characterized in that it shows one or more substitutions, deletions and/or insertions in comparison to the corresponding sequence from which it is derived and wherein these substitutions, deletions and/or insertions occur at one or more of the positions corresponding to positions 282, 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315 in the amino acid sequence shown in SEQ ID NO:1. "Corresponding to" these positions means corresponding to any of these positions in a related sequence.

As disclosed herein, the mevalonate diphosphate decarboxylase from which the variant is derived is a mevalonate diphosphate decarboxylase which shows the amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 40%, preferably at least 50% and even more preferably at least 60%, or most preferred at least 90% sequence identity to SEQ ID NO:1.

Accordingly, there is disclosed a variant of a mevalonate diphosphate decarboxylase having an amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 40%, 50%, 60% or 90% sequence identity to SEQ ID NO:1, wherein one or more amino acid residues at a position selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 282, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence, are substituted with another amino acid residue or deleted or show an insertion at at least one of these positions and wherein said mevalonate diphosphate decarboxylase has improved activity in converting 3-phosphonoxyisovalerate into isobutene. In a preferred embodiment the deletion, insertion or substitution is at a position selected from the group consisting of positions 9, 11, 42, 43, 45, 66, 77, 116, 118, 120, 121, 123, 129, 134, 159, 160, 173, 177, 186, 251, 253, 282, 293, 297, 299, 303, 307 and 308.

[0016] The present inventors have found that the activity of a mevalonate diphosphate decarboxylase to catalyze the second step of the above described conversion of 3-hydroxyisovalerate into isobutene, i.e. the conversion of 3-phosphonoxyisovalerate into isobutene, can dramatically be improved by mutating the MDP decarboxylase enzyme at certain positions. They used as a model enzyme the enzyme from *S. mitis*, the sequence of which is shown in SEQ ID NO: 1. The identified single mutations lead to an increase of activity of up to more than 300% when compared to the unmutated sequence of the *S. mitis* enzyme (represented by SEQ ID NO: 1). Combining mutations led to a further increase of up to 750% and the identification of additional mutations in addition to these combinations allowed to achieve a further 16-fold increase.

In particular, the present inventors found that substitutions at positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 282, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, or 315 or substitutions at combinations of these positions lead to a drastic increase in the enzyme's ability to convert 3-

phosphonoxyisovalerate into isobutene.

As indicated above, the variants of an MDP decarboxylase according to the present invention are characterized in that they show an increased activity in converting 3-phosphonoxyisovalerate into isobutene when compared to the MDP decarboxylase from which they are derived. Thus, in the case where the variant is derived from the MDP decarboxylase of *S. mitis* having the amino acid sequence shown in SEQ ID NO: 1, the variant shows an increased activity in converting 3-phosphonoxyisovalerate into isobutene when compared to the MDP decarboxylase shown in SEQ ID NO: 1. When the variant is derived from an MDP decarboxylase which is structurally related to the MDP decarboxylase of *S. mitis* as defined herein above, the variant shows an increased activity in converting 3-phosphonoxyisovalerate into isobutene when compared to the corresponding starting sequence into which the corresponding mutations have been introduced. In a particularly preferred embodiment such variants show also an increased activity in converting 3-phosphonoxyisovalerate into isobutene when compared to the MDP decarboxylase shown in SEQ ID NO: 1. The activity of converting 3-phosphonoxyisovalerate into isobutene may be determined by methods known to the person skilled in the art. In one embodiment, this activity is determined as described in the Examples appended hereto. In a particular embodiment this activity can be measured by using in particular the following assay:

[0017] A nucleic acid molecule encoding the respective enzyme is transformed into *E. coli*, such as *E. coli* BL21DE3 and the enzyme is expressed in *E. coli*. The activity of the enzyme can then be measured using either

- crude cell lysate (i.e. the cell culture is centrifuged, the cell pellet is resuspended in a hypotonic buffer, is sonicated or not but is not centrifuged); or
- the soluble fraction (i.e. the supernatant obtained following centrifugation of the crude cell lysate); or
- purified protein (i.e. recombinant protein which is present in the soluble fraction and which is, for example, trapped on a resin using chromatography).

The activity of the expressed enzyme is tested as follows:

A reaction mixture is prepared in glass GC vials by mixing the crude cell lysate, the soluble fraction or the purified protein as described above with 3-phosphonoxyisovalerate in 50 mM Tris pH 7 final concentration. ATP, $MgCl_2$ and KCl are provided as cofactors.

The vials are hermetically sealed and the reaction mixture is incubated for an appropriate time (e.g. 24h) at 37°C. The isobutene produced by the reaction is determined by gas chromatography by injecting the gas phase obtained from the vial into a gas chromatograph. Controls in the assay are culture with untransformed bacteria, bacteria transformed with an empty expression vector and bacteria expressing the corresponding starting enzyme, e.g. the *S. mitis* enzyme showing the amino acid sequence as shown in SEQ ID NO:1.

The 3-phosphonoxyisovalerate used in the assay may be prepared in different ways. One possibility is to provide this compound by the following reaction:

0.063 mg/ml of purified *Thermophilus acidophilum* MDP decarboxylase is mixed with 50 mM hydroxyisovalerate, 40 mM ATP in a 50 mM Tris-Cl pH 7, 20 mM KCl, 20 mM $MgCl_2$ and incubated for 24 hours at 45°C. This enzymatically prepared 3-phosphonoxyisovalerate substrate is used in the reaction mixture.

Another, preferred possibility is that the 3-phosphonoxyisovalerate used in the assay is chemically synthesized. The synthesis scheme is shown in Figure 9.

If chemically synthesized 3-phosphonoxyisovalerate is used in the assay, the concentration is preferably between 3 and 8 mM, preferably it is 5 mM.

In a preferred assay for testing the enzyme activity purified enzyme is used. In this case, the reaction mixture contains

500 µg purified enzyme in 50 mM Tris HCl pH 7.5 and
3-phosphonoxyisovalerate (provided as described above)

It is made sure that cofactors such as ATP, $MgCl_2$ and KCl are present. The volume is adjusted to 500 µl with 50 mM Tris HCl pH 7.5 and the reaction is incubated for 15 hours at 37°C. Subsequently the isobutene produced is determined by gas chromatography.

In one embodiment, the 3-phosphonoxyisovalerate is prepared enzymatically as described above.

In a particularly preferred embodiment the 3-phosphonoxyisovalerate is chemically synthesized. In this case, the reaction mixture contains:

200 µg purified enzyme

3-phosphonoxyisovalerate

5mM ATP, 20mM KCl, 10mM MgCl₂ and 50 mM Tris-Cl pH 7.5

In order to test the activity of the enzyme varying concentrations of 3-phosphonoxyisovalerate are used, preferably the following concentrations: 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, 10, mM, 20 mM, 40 mM, 80 mM and 160 mM. It is particularly preferred that the concentration of 3-phosphonoxyisovalerate is 5 mM. The volume is adjusted to 500 µl with 50 mM Tris HCl pH 7.5 and the reaction is incubated for 15 hours at 37°C. Subsequently the isobutene produced is determined by gas chromatography.

[0018] Examples for such an assay to determine the activity of an enzyme to convert 3-phosphonoxyisovalerate into isobutene are given in the Example section below in Example 1.

[0019] In the context of the present invention, an "improved activity" means that the activity of the MDP decarboxylase in question is at least 10%, preferably at least 20%, more preferably at least 30% or 50%, even more preferably at least 70% or 80% and particularly preferred at least 90% or 100% higher than that of the MDP decarboxylase from which the variant is derived, preferably higher than that of the MDP decarboxylase of *S. mitis* represented by SEQ ID NO:1. In even more preferred embodiments the improved activity may be at least 150%, at least 200%, at least 300%, at least 750% or at least 1000% higher than that of the corresponding MDP decarboxylase from which the variant is derived, preferably higher than that of the MDP decarboxylase of *S. mitis* represented by SEQ ID NO:1. In a particularly preferred embodiment, the activity is measured by using an assay with purified enzyme and chemically synthesized 3-phosphonoxyisovalerate as described herein-above. The improved activity of a variant can be measured as a higher isobutene production in a given time under defined conditions, compared with the parent enzyme. This improved activity can result from a higher kcat value. It can also result from a lower Km value. It can also result from a higher kcat/Km value. The degree of improvement can be measured as the improvement in isobutene production. The degree of improvement can also be measured in terms of kcat improvement, of kcat/Km improvement, or in terms of Km decrease.

[0020] According to one embodiment, the MDP decarboxylase of the present invention has an amino acid sequence in which

- (1) an amino acid residue at position 1 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine; and/or
- (2) 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 in a related sequence, is deleted or substituted with histidine; and/or
- (3) an amino acid residue at position 9 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine; and/or
- (4) an amino acid residue at position 11 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine, glutamic acid or phenylalanine, preferably cysteine; and/or
- (5) an amino acid residue at position 16 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine; and/or
- (6) an amino acid residue at position 23 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine; and/or
- (7) 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 in a related sequence, is deleted or substituted with arginine, serine, or leucine; and/or

- (8) an amino acid residue at position 28 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with lysine or alanine; and/or
- (9) an amino acid residue at position 31 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with serine; and/or
- (10) an amino acid residue at position 42 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine or leucine; and/or
- (11) an amino acid residue at position 43 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine; and/or
- (12) an amino acid residue at position 45 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine, phenylalanine, methionine or valine, preferably leucine; and/or
- (13) an amino acid residue at position 53 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with valine; and/or
- (14) an amino acid residue at position 57 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with serine; and/or
- (15) an amino acid residue at position 58 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with threonine; and/or
- (16) an amino acid residue at position 66 in the amino acid sequence shown in SEQ ID NO:1 at a position corresponding to this position in a related sequence, is deleted or substituted with histidine; and/or
- (17) an amino acid residue at position 75 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with isoleucine; and/or
- (18) an amino acid residue at position 77 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with asparagine or arginine; and/or
- (19) 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 in a related sequence, is deleted or substituted with glycine; and/or
- (20) 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 in a related sequence, is deleted or substituted with glutamine; and/or
- (21) an amino acid residue at position 87 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or
- (22) an amino acid residue at position 91 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with histidine; and/or
- (23) an amino acid residue at position 105 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine; and/or
- (24) an amino acid residue at position 111 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with methionine; and/or
- (25) an amino acid residue at position 116 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with arginine, isoleucine, leucine, serine or methionine, preferably arginine or isoleucine; and/or
- (26) an amino acid residue at position 118 in the amino acid sequence shown in SEQ ID NO:1 at a position

corresponding to this position in a related sequence, is deleted or substituted with leucine or tryptophane; and/or

(27) an amino acid residue at position 120 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with asparagine, leucine, arginine, isoleucine or valine, preferably asparagine, leucine, arginine or isoleucine; and/or

(28) an amino acid residue at position 121 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with arginine, leucine, tryptophan, phenylalanine, tyrosine, asparagine or lysine, preferably arginine or phenylalanine; and/or

(29) the amino acid residue at position 122 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is substituted with methionine or tyrosine; and/or

(30) an amino acid residue at position 123 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with methionine or arginine; and/or

(30) 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 in a related sequence, is deleted or substituted with proline or valine; and/or

(32) an amino acid residue at position 134 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glycine; and/or

(33) an amino acid residue at position 139 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine or alanine; and/or

(34) an amino acid residue at position 141 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with proline, cysteine, glycine or threonine; and/or

(35) an amino acid residue at position 142 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine; and/or

(36) an amino acid residue at position 159 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine; and/or

(37) an amino acid residue at position 160 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with valine; and/or

(38) an amino acid residue at position 161 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with arginine; and/or

(39) an amino acid residue at position 164 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamine; and/or

(40) 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 in a related sequence, is deleted or substituted with serine; and/or

(41) an amino acid residue at position 173 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine; and/or

(42) an amino acid residue at position 177 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with valine or cysteine, preferably valine; and/or

(43) an amino acid residue at position 179 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with lysine or leucine; and/or

(44) an amino acid residue at position 180 in the amino acid sequence shown in SEQ ID NO:1 or at a position

corresponding to this position in a related sequence, is deleted or substituted with proline; and/or

(45) an amino acid residue at position 182 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or

(46) an amino acid residue at position 186 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with histidine, leucine, valine, isoleucine or asparagine; and/or

(47) an amino acid residue at position 188 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine; and/or

(48) 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 in a related sequence, is deleted or substituted with aspartic acid; and/or

(49) 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 in a related sequence, is deleted or substituted with leucine; and/or

(50) an amino acid residue at position 205 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with histidine; and/or

(51) 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 in a related sequence, is deleted or substituted with leucine; and/or

(52) an amino acid residue at position 215 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine; and/or

(53) an amino acid residue at position 221 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or

(54) an amino acid residue at position 227 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with lysine; and/or

(55) an amino acid residue at position 231 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamine or leucine; and/or

(56) 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 in a related sequence, is deleted or substituted with arginine, glutamic acid or lysine; and/or

(57) 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 in a related sequence, is deleted or substituted with methionine or isoleucine; and/or

(58) an amino acid residue at position 242 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine or glutamic acid; and/or

(59) an amino acid residue at position 246 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or

(60) an amino acid residue at position 248 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with threonine; and/or

(61) an amino acid residue at position 251 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with methionine, phenylalanine or valine, preferably methionine; and/or

- (62) an amino acid residue at position 252 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or
- (63) an amino acid residue at position 253 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with valine or isoleucine; and/or
- (64) an amino acid residue at position 255 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamic acid; and/or
- (65) an amino acid residue at position 258 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with leucine; and/or
- (66) an amino acid residue at position 264 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with glutamine; and/or
- (67) an amino acid residue at position 267 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with arginine; and/or
- (68) an amino acid residue at position 279 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with alanine; and/or
- (69) an amino acid residue at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine, serine, glutamic acid, glycine, glutamine, threonine, valine, alanine or aspartic acid; and/or
- (70) an amino acid residue at position 291 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with aspartic acid; and/or
- (71) an amino acid residue at position 293 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with phenylalanine or tryptophan; and/or
- (72) an amino acid residue at position 297 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with cysteine or leucine, preferably cysteine; and/or
- (73) an amino acid residue at position 299 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with proline or lysine; and/or
- (74) an amino acid residue at position 303 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with methionine; and/or
- (75) an amino acid residue at position 307 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with histidine; and/or
- (76) an amino acid residue at position 308 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with serine; and/or
- (77) an amino acid residue at position 315 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, is deleted or substituted with serine.

[0021] The invention also relates to variants as defined in (1) to (77) hereinabove, wherein the amino acid residue indicated as substituting the amino acid residue at the position in SEQ ID NO: 1 is not that particular amino acid residue but an amino acid residue which is conservative in relation to the indicated substituting amino acid. Whether an amino acid is conservative with respect to another amino acid can be judged according to means and methods known in the art. One possibility is the PAM 250 matrix; alternatively, the Blosum Family

Matrices can be used.

[0022] In one embodiment the present invention relates to a variant of a MDP decarboxylase having an amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 60% or 90% sequence identity to SEQ ID NO:1, wherein the amino acid residue at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is substituted with another amino acid residue or is deleted. In a preferred embodiment the present invention relates to such a variant in which at least one further amino acid residue is substituted or deleted at a position selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 45, 53, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 141, 159, 161, 173, 177, 180, 215, 238, 241, 242, 248, 251, 253, 264, 279, 291, 293, 297, 299, 303, 307, 308 and 315, even more preferably selected from the group consisting of positions 9, 11, 42, 45, 116, 118, 120, 121, 122, 123, 129, 177, 251, 253, 264, 293, 297 and 303.

[0023] In one particular embodiment, the amino acid residues at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is substituted with cysteine, serine, glutamic acid, glycine, glutamine, threonine, valine, alanine or aspartic acid, preferably with cysteine. In another particular embodiment, the substitutions at any one of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315 are as indicated herein-above.

[0024] There is also disclosed a variant of a MDP decarboxylase having an amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 40%, 50%, 60% or 90% sequence identity to SEQ ID NO:1, wherein the amino acid residues at position 121 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is deleted or substituted with another amino acid residue, preferably arginine, leucine, lysine or phenylalanine. There is also disclosed such a variant in which at least one further amino acid residue is deleted or substituted at a position selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 282, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 11, 16, 24, 28, 45, 53, 80, 91, 105, 116, 118, 120, 123, 141, 159, 161, 173, 177, 180, 215, 238, 241, 242, 248, 258, 279, 282, 291, 297, 299, 303, 307, 308 and 315. The substitutions at any of these positions are preferably those as listed herein-above.

[0025] The present invention also relates to a variant of a MDP decarboxylase having an amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 60% or 90% sequence identity to SEQ ID NO:1, wherein the amino acid residues at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is deleted or substituted with another amino acid residue, wherein the amino acid residue at position 121 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is deleted or substituted with another amino acid residue and wherein at least one further amino acid residue is substituted at a position selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 11, 16, 24, 28, 45, 53, 80, 91, 105, 116, 118, 120, 123, 141, 159, 161, 173, 177, 180, 215, 238, 241, 242, 248, 258, 279, 291,

297, 299, 303, 307, 308 and 315.

[0026] In another particular embodiment, the amino acid residue at position 121 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is substituted with arginine, leucine, lysine and phenylalanine. In a further preferred embodiment, the substitutions at any one of the positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315 are those as indicated herein-above.

[0027] In a preferred embodiment the variant according to the invention is characterized in that it contains at least three deletions and/or substitutions, wherein one deletion/substitution is at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence, another deletion/substitution is at position 121 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence and at least one further deletion/substitution is at a position selected from the group consisting of positions 11, 45, 116, 120 or 177 of SEQ ID NO: 1 or a position corresponding to any of these positions.

It is preferred that the following positions in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence in a related sequence as defined above are at least modified by deletion or substitution:

45, 121 and 282; or

11, 121 and 282; or

116, 121 and 282; or

121, 177 and 282; or

120, 121 and 282; or

173, 282 and 297.

The substitutions at the indicated positions are preferably those as indicated herein-above.

[0028] Particularly preferred variants with three mutations show the following substitutions in SEQ ID NO:1:

E45L-Y121R-K282C

K282C-Y121R-Y11 E

K116I-Y121R-K282C

Y121R-E177V-K282C

A120R-Y121L-K282C

M173C-K282C-F297L

or the corresponding substitutions at the corresponding positions in a structurally related sequence as defined above.

[0029] The present invention also relates to a variant of a MDP decarboxylase having an amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 60% or 90% sequence identity to SEQ ID NO:1, wherein the amino acid residues at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is substituted with another amino acid residue, wherein the amino acid residues at position 297 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is substituted with another amino acid residue and

wherein the amino acid residues at position 173 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is substituted with another amino acid residue. In a particular embodiment, the amino acid residues at position 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is substituted with cysteine, serine, glutamic acid, glycine, glutamine, threonine, valine, alanine or aspartic acid, preferably with cysteine. In another particular embodiment, the amino acid residue at position 297 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to this position in a related sequence is substituted with leucine. In a further embodiment the amino acid residues at position 173 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding in a related sequence to this position is substituted with cysteine.

[0030] There is also disclosed a variant which is characterized in that it contains at least four deletions and/or substitutions at positions selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 9, 11, 16, 24, 28, 45, 53, 80, 91, 105, 118, 121, 123, 141, 159, 161, 173, 177, 180, 215, 238, 241, 242, 248, 258, 279, 282, 291, 297, 299, 303, 307, 308 and 315 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence as defined above. Preferably, one of the deletions/substitutions is at position 303 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a methionine. In another embodiment the substitutions at the remaining three positions are effected at positions selected from the group consisting of positions 45, 121, 173, 282, 307 and 308 or at a position corresponding to any of these positions in a related sequence as defined above.

It is preferred that the following positions in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above are at least modified by deletion or substitution:

121, 282, 303 and 308; or

173, 303, 307 and 308; or

45, 173, 282 and 303.

Preferably, the substitution at position 45 is valine, the substitution at position 121 is an arginine, the substitution at position 173 or 282 is a cysteine, the substitution at position 307 is a histidine and the substitution at position 308 is a serine. Most preferred are the following combinations of substitutions: Y121R-K282C-L303M-T308S; M173C-L303M-K307H-T308S; E45V-M173C-K282C-L303M or the corresponding substitutions at the corresponding positions in a structurally related sequence as defined above.

[0031] There is also disclosed a variant which is characterized in that it contains at least five deletions and/or substitutions at positions selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 9, 11, 16, 24, 28, 45, 53, 80, 91, 105, 118, 121, 123, 141, 159, 161, 173, 177, 180, 215, 238, 241, 242, 248, 258, 279, 282, 291, 297, 299, 303, 307, 308 and 315 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence as defined above. Preferably, one of these substitutions is at position 303 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a methionine. In another embodiment the deletions/substitutions at the remaining three positions are effected at positions selected from the group consisting of positions 9, 11, 118, 121, 159, 173, 282, 307 and 308 or at a position corresponding to any of these positions in a related sequence as defined above.

It is preferred that the following positions in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above are at least modified by deletion or substitution:

121, 173, 282, 303 and 308; or

159, 173, 303, 307 and 308; or

9, 11, 303, 307 and 308; or

118, 121, 173, 282 and 303.

Preferably the substitution at position 9 is leucine, the substitution at position 11 is phenylalanine, the substitution at position 118 is leucine, the substitution at position 121 is an arginine, the substitution at position 159 is leucine, the substitution at position 173 or 282 is a cysteine, the substitution at position 307 is a histidine and the substitution at position 308 is a serine.

Particularly preferred variants with five mutations show the following substitutions in SEQ ID NO:1:

Y121R-M173C-K282C-L303M-T308S

E159L-M173C-L303M-K307H-T308S

R9L-Y11F-L303M-K307H-T308S

C118L-Y121R-M173C-K282C-L303M

or the corresponding substitutions at the corresponding positions in a structurally related sequence as defined above.

[0032] There is also disclosed a variant which is characterized in that it contains at least six deletions and/or substitutions at positions selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 16, 24, 28, 45, 53, 80, 91, 105, 118, 121, 123, 141, 159, 161, 173, 177, 180, 215, 238, 241, 242, 248, 258, 279, 282, 291, 297, 299, 303, 307, 308 and 315 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence as defined above. Preferably, one of these deletions/substitutions is at position 282 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a cysteine. In another embodiment, one of these deletions/substitutions is at position 173 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a cysteine. In another embodiment the deletions/substitutions at the remaining four positions are effected at positions selected from the group consisting of positions 45, 121, 159, 215, 297, 303 and 308 or at a position corresponding to any of these positions in a related sequence as defined above.

It is preferred that the following positions in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above are at least modified by deletion or substitution:

121, 159, 173, 282, 303 and 308; or

121, 159, 173, 215, 282 and 303; or

45, 159, 173, 282, 297 and 308.

Preferably, the substitution at position 45 is leucine, the substitution at position 121 is an arginine, the substitution at position 159 is leucine, the substitution at position 215 is alanine, the substitution at position 297 is leucine, the substitution at position 303 is a methionine and the substitution at position 308 is a serine. Most preferred are the following combinations of substitutions:

Particularly preferred variants with six mutations show the following substitutions in SEQ ID NO:1:

Y121R-E159L-M173C-K282C-L303M-T308S

Y121R-E159L-M173C-V215A-K282C-L303M

E45L-E159L-M173C-K282C-F297L-T308S

or the corresponding substitutions at the corresponding positions in a structurally related sequence as defined above.

[0033] There is also disclosed a variant which is characterized in that it contains at least seven substitutions at positions selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 16, 24, 28, 45, 53, 80, 91, 105, 118, 121, 123, 141, 159, 161, 173, 177, 180, 215, 238, 241, 242, 248, 258, 279, 282, 291, 297, 299, 303, 307, 308 and 315 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence as defined above. Preferably, one of these deletions/substitutions is at position 282 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a cysteine. In another embodiment, one of these deletions/substitutions is at position 173 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a cysteine. In another embodiment, one of these deletions/substitutions is at position 121 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by an arginine. In another embodiment, one of these deletions/substitutions is at position 303 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a methionine. In another embodiment the deletions/substitutions at the remaining three positions are effected at positions selected from the group consisting of positions 45, 118, 159, 177, 242, 297, 307, 308 and 315 or at a position corresponding to any of these positions in a related sequence as defined above.

It is preferred that the following positions in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above are at least modified by deletion or substitution:

121, 159, 173, 282, 303, 307 and 308; or

45, 121, 159, 173, 282, 303 and 308; or

121, 159, 173, 282, 297, 303 and 308; or

118, 121, 159, 173, 282, 303 and 308; or

121, 159, 173, 177, 282, 303 and 308; or

121, 159, 173, 242, 282, 303 and 308; or

118, 121, 159, 173, 282, 303 and 315; or

118, 121, 159, 173, 177, 282 and 303;

45, 121, 173, 282, 297, 303 and 308; or

45, 118, 121, 173, 282, 303 and 308; or

45, 121, 159, 173, 282, 297 and 303.

Preferably, the substitution at position 45 is valine, the substitution at position 118 is leucine or tryptophan, the substitution at position 159 is leucine, the substitution at position 177 is cysteine, the substitution at position 242 is alanine, the substitution at position 297 is leucine, the substitution at position 307 is a histidine and the substitution at position 308 or 315 is a serine. Most preferred are the following combinations of substitutions:

Particularly preferred variants with seven mutations show the following substitutions in SEQ ID NO:1:

Y121R-E159L-M173C-K282C-L303M-K307H-T308S

E45V-Y121R-E159L-M173C-K282C-L303M-T308S

Y121R-E159L-M173C-K282C-F297L-L303M-T308S

C118L-Y121R-E159L-M173C-K282C-L303M-T308S

Y121R-E159L-M173C-E177C-K282C-L303M-T308S

Y121R-E159L-M173C-T242A-K282C-L303M-T308S

C118L-Y121R-E159L-M173C-K282C-L303M-G315S

C118W-Y121R-E159L-M173C-E177C-K282C-L303M

C118L-Y121R-E159L-M173C-E177C-K282C-L303M

E45V-Y121 R-M173C-K282C-F297L-L303M-T308S

E45V-C118L-Y121R-M173C-K282C-L303M-T308S

E45V-Y121R-E159L-M173C-K282C-F297L-L303M

or the corresponding substitutions at the corresponding positions in a structurally related sequence as defined above.

[0034] There is also disclosed a variant which is characterized in that it contains at least eight or at least nine deletions and/or substitutions at positions selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 16, 24, 28, 45, 53, 80, 91, 105, 118, 121, 123, 141, 159, 161, 173, 177, 180, 215, 238, 241, 248, 258, 279, 282, 291, 297, 299, 303, 308 and 315 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence as defined above. Preferably, one of these deletions/substitutions is at position 282 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a cysteine. In another embodiment, one of these deletions/substitutions is at position 173 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a cysteine. In another embodiment, one of these deletions/substitutions is at position 121 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by an arginine. In another embodiment, one of these deletions/substitutions is at position 303 of SEQ ID NO:1 or at a position corresponding to this position in a related sequence as defined above. More preferably, this position is replaced by a methionine. In another embodiment the deletions/substitutions at the remaining four and five positions, respectively, are effected at positions selected from the group consisting of positions 24, 45, 80, 118, 123, 159, 177, 215, 258, 297, 308 and 315 or at a position corresponding to any of these positions in a related sequence as defined above.

[0035] It is preferred that the following positions in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above are at least modified by deletion or substitution:

80, 121, 159, 173, 282, 303, 308 and 315; or

24, 121, 159, 123, 173, 258, 282 and 303; or

118, 121, 159, 173, 177, 215, 282 and 303; or

24, 118, 121, 159, 173, 177, 282 and 303; or

45, 80, 121, 173, 282, 297, 303 and 308; or

45, 121, 159, 173, 177, 282, 303 and 308; or

118, 121, 159, 173, 177, 282, 297, 303 and 308; or

118, 121, 159, 173, 177, 215, 282, 297 and 303; or

80, 118, 121, 159, 173, 177, 282, 303 and 315; or

24, 45, 121, 123, 173, 282, 297, 303 and 308; or

45, 121, 159, 173, 177, 215, 258, 282 and 303.

Preferably, the substitution at position 24 is arginine, the substitution at position 45 is valine or leucine, the substitution at position 80 is glycine, the substitution at position 118 is leucine or tryptophan, the substitution at position 123 is arginine, the substitution at position 159 is leucine, the substitution at position 177 is cysteine, the substitution at position 215 is alanine, the substitution at position 258 is leucine, the substitution at position 297 is leucine and the substitution at position 308 or 315 is a serine.

Particularly preferred variants with eight or nine mutations show the following substitutions in SEQ ID NO:1:

D80G-Y121 R-E159L-M173C-K282C-L303M-T308S-G315S

K24R-121R-E159L-K123R-M173C-M258L-K282C-L303M

C118L-Y121R-E159L-M173C-E177C-V215A-K282C-L303M

K24R-C118L-Y121R-E159L-M173C-E177C-K282C-L303M

E45V-D80G-Y121R-M173C-K282C-F297L-L303M-T308S

E45L-Y121R-E159L-M173C-E177C-K282C-L303M-T308S

C118L-Y121R-E159L-M173C-E177C-K282C-F297L-L303M-T308S

C118L-Y121R-E159L-M173C-E177C-V215A-K282C-F297L-L303M

D80G-C118L-Y121R-E159L-M173C-E177C-K282C-L303M-G315S

K24R-E45V-Y121R-K123R-M173C-K282C-F297L-L303M-T308S

E45V-Y121R-E159L-M173C-E177C-V215A-M258L-K282C-L303M

or the corresponding substitutions at the corresponding positions in a structurally related sequence as defined above.

[0036] There is also disclosed a variant which is characterized in that it contains at least ten substitutions at positions selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 16, 24, 28, 45, 53, 91, 105, 118, 121, 141, 159, 161, 173, 177, 180, 215, 238, 241, 248, 279, 282, 291, 297, 299, 303 and 308 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence as defined above. In one embodiment the deleted/substituted positions are positions 45, 118, 121, 159, 173, 215, 282, 297, 303 and 308. Preferably, the substitution at position 45 is valine, the substitution at position 118, 159 and 297 is leucine, the substitution at position 121 is arginine, the substitution at position 173 or 282 is cysteine, the substitution at position 215 is

alanine, the substitution at position 303 is methionine and the substitution at position 308 is a serine. Thus, a particularly preferred variant with ten mutations shows the following substitutions in SEQ ID NO:1:

E45V-C118L-Y121R-E159L-M173C-V215A-K282C-F297L-L303M-T308S

or the corresponding substitutions at the corresponding positions in a structurally related sequence as defined above.

[0037] There is also disclosed a variant which is characterized in that it contains at least eleven deletions and/or substitutions at positions selected from the group consisting of positions 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315, preferably selected from the group consisting of positions 16, 24, 28, 53, 91, 105, 118, 121, 141, 159, 161, 173, 177, 180, 238, 241, 248, 279, 282, 291, 297, 299, 303 and 308 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence as defined above. In one embodiment the deleted/substituted positions are positions 24, 118, 121, 159, 173, 177, 282, 291, 297, 303 and 308. Preferably, the substitution at position 24 or 121 is arginine, the substitution at position 118, 159 and 297 is leucine, the substitution at position 173, 177 or 282 is cysteine or valine, the substitution at position 291 is aspartate, the substitution at position 303 is methionine and the substitution at position 308 is a serine. Thus, a particularly preferred variant with eleven mutations shows the following substitutions in SEQ ID NO:1:

K24R-C118L-Y121R-E159L-M173C-E177C-K282C-E291 D-F297L-L303M-T308S or the corresponding substitutions at the corresponding positions in a structurally related sequence as defined above.

A mutant containing these 11 substitutions is also referred to as "F9" in the context of the appended Example section.

[0038] There is also disclosed a variant which is characterized in that it contains at least ten, preferably at least eleven deletions and/or substitutions at positions selected from the group consisting of position 24, 118, 121, 159, 173, 177, 282, 291, 297, 303 and 308 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above, preferably substitutions as in the variant F9, and that it contains in addition at least one further deletion/substitution at a position selected from the group consisting of positions 16, 28, 53, 91, 105, 141, 161, 180, 238, 241, 248, 279, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 299 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above. Preferably substitutions at any of these positions are as defined herein-above.

In one embodiment the at least one further deletion/substitution is at positions 16 and 105 or at positions 16 and 141 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 141 and 241 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 141, 241 and 248 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 141, 241 and 248 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 91, 141, 241 and 248 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 91, 141, 241, 248 and 299 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 91, 141, 241, 248, 299 and 28 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 91, 141, 241, 248, 299 and 28 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 141, 241, 248 and 28 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 141, 241, 248, 28 and 180 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 141, 241, 248, 28, 53 and 180. in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above

In another embodiment the at least one further deletion/substitution is at positions 16, 141, 241, 248, 28, 180 and 238 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 141, 241, 248, 28, 180 and 279 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

In another embodiment the at least one further deletion/substitution is at positions 16, 141, 241, 248, 28, 180 and 161 in SEQ ID NO:1 or the positions corresponding to any of these positions in a related sequence as defined above.

Particularly preferred variants show at least ten, preferably at least eleven deletions and/or substitutions at positions selected from the group consisting of position 24, 118, 121, 159, 173, 177, 282, 291, 297, 303 and 308, preferably substitutions as in the variant F9, in SEQ ID NO:1 or the corresponding substitutions at the corresponding positions in a structurally related sequence as defined above, and show in addition the following substitution(s) in SEQ ID NO:1:

S141P

S141T

S105A

Q299K

I16L

S248T

K241M

I16L-S105A

S141P-K241M-S248T

I16L-R91H-S141P-K241 M-S248T

I16L-S141P

116L-R91 H-S141 P-K241M-S248T Q299K

116L-S141P-K241M

I16L-S141P-K241 M-S248T

I16L-R91H-S141P-K241M-S248T-Q299K-M28K

I16L-R91H-S141P-K241M-S248T-Q299K-M28A

I16L-R91 H-S141P-K241M-S248T-Q299K-K180P

I16L-S141P-K241I-S248T

I16L-S141 P-K241I-S248T-M28K

I16L-S141P-K241I-S248T-M28K-K180P

I16L-S141 P-K241I-S248T-M28K-T53V-K180P

I16L-S141P-K241I-S248T-M28K-K180P-A238K

I16L-S141P-K241I-S248T-M28K-K180P-A238R

I16L-S141 P-K241I-S248T-M28K-K180P-C282V

I16L-S141 P-K241I-S248T-M28K-K180P-P279A

I16L-S141 P-K241I-S248T-M28K-K180P-Y161R

D2H

M42L-D87E-S139C-R186L-K231Q

E164Q-R186V-D252E

D87E-S139C-R186L-K231Q

R186V-Q267R

S139C-R186I

L111 M-F122Y-R186L

M75I-R186V

S139A-S141C

K179K-R186V

R186V

A57S-A58T-K77R-R186V

L111M-R186L

R186L

A31S-R186V

S139A-S141G

M75I-R186L-S308T

R186L-S308T

R186I

L111M-R186V-S308T

R186V-D221E

L111M-R186V

M1L-L111M-R186V-S308T

R186N

R24S-G86Q-R186I

I16L-S141P-K241I-K180P-E227K

I16L-S141P-K241I-K180P-D291E-M303L

S141P-K241I-S248T-K180P-R24K

I16L-S141P-K241I-S248T-L297F

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-A246E

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-T242E

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-Y255E

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-T198D

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-K23L

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-K179L

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-K231L

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-P182E

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-A238E

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-K208L

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-R204L

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-R24L

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F

I16L-S141P-K241I-K180P-D291E

I16L-S141P-K241I-S248T-K180P-Q267R-R24K-L118C-L159E

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-S142A

I16L-S141P-K241I-S248T-M28K-K180P-L159E-D291E

I16L-S141P-K241I-S248T-K180P-G166S-R24K

I16L-S141P-K241I-S248T-K180P-R24K

I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-Q205H

or the corresponding substitution(s) at the corresponding positions in a structurally related sequence as defined above.

[0039] The present inventors also constructed a computer 3D model of the *S. mitis* enzyme represented by the amino acid sequence shown in SEQ ID NO: 1 (see Figure 6) in order to locate the mutated residues in the three dimensional structure. The structure of the enzyme is characterized by a cone-shaped fold, where the N-terminal region is oriented orthogonally against the relatively plane C-terminal region comprising five alpha helices. The active site is a positively charged cleft formed between the N and C terminal domains with an ATP binding P-loop nearby. The 3D structure of the *S. mitis* enzyme is extremely similar to the 3D structure of the MDP decarboxylase of *S. pyogenes* (see Figure 6), the amino acid sequence of which shows 69% sequence identity to the *S. mitis* enzyme (SEQ ID NO:1). The 3D structure is also very similar to that reported in Byres et al. (J.

Mol. Biol. 371 (2007), 540-553) for the MDP decarboxylases of *S. brucei* and *S. aureus*. Figure 7 shows the sequence of the MDP decarboxylase in which the secondary structure (β -sheets and α -helices) as well as some of the mutated residues are highlighted.

[0040] It has been demonstrated that the mutations identified in the present invention have a direct effect on the rate of production of isobutene from 3-phosphonoxyisovalerate by the enzyme; however it is possible that these mutations confer to the enzyme other properties that have not been fully identified yet. For example, several substitutions replace wild type residues with cysteine residues (Y11, M173 and K282). In the improved mutants residues 2B4, M173C and K282C are close enough in the 3D modeled structure to suggest that the creation of a disulphide bridge is possible. Such a bond could stabilize the overall structure of the enzyme. To support this, some preliminary data indicates that some mutants are more resistant to thermal denaturation than the wild type enzyme.

Similarly these mutations may have an effect on the stability to pH, optimal temperature and the oligomeric state of the proteins. Preliminary data collected on the effect of pH on isobutene production indicate that some mutant variants are more active at acidic pH compared to the wild type enzyme.

[0041] One group of the identified positions are located in the N-terminal domain of the protein, in particular positions 1, 2, 9, 11, 42, 43, 45, 57, 58, 75, 77, 80, 86, 87, 91, 116, 118, 120, 121, 122, 123, 129 and 134.

An analysis revealed that residues 77, 80, 116, 118, 120, 121, 122, 123, 129 and 134 are located on the alpha helices N° 1, 2 and 3 in the N-terminal domain and are situated most distally from the substrate binding pocket. Residues 9, 11, 42, 43, 45 and 91 are located in close proximity to these alpha helices and in particular, they are located in the beta sheet on beta strands 1 and 4 that are directly adjacent to each other (see Figure 8). Thus, all these residues are located in the N-terminus, are located close to each other in the 3D structure and do not show any structural proximity to the active site of the enzyme. Thus,

there is also disclosed a variant of a mevalonate diphosphate decarboxylase having an amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 40%, 50%, 60% or 90% sequence identity to SEQ ID NO:1, wherein one or more amino acid residues at a position selected from the group consisting of positions 9, 11, 42, 43, 45, 77, 80, 91, 116, 118, 120, 121, 122, 123, 129 and 134 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions, are substituted with another amino acid residue and wherein said mevalonate diphosphate decarboxylase has improved activity in converting 3-phosphonoxyisovalerate into isobutene.

[0042] Another group of the identified positions, in particular positions 159, 160, 164, 166, 173, 177, 179, 198, 204, 205, 208, 221, 227, 231, 238, 241, 242, 246, 248, 251, 252, 253, 255, 258, 264, 267, 282, 291, 293, 297, 299, 303, 307 and 308, are located in the C-terminal domain and most of these positions are located in alpha helices and in beta strands, in particular in alpha helices 8 and 9 and in beta strands 9 to 12. It is assumed that mutations at positions 238, 241, 242, 248, 253, 258, 264, 291, 293, 297, 299, 303, 307 and 308 (i.e. in the alpha helix no° 9 and in beta-strand no° 12) might create a more stable conformation that stabilizes the whole structure. Thus,

there is also disclosed a variant of a mevalonate diphosphate decarboxylase having an amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 40%, 50%, 60% or 90% sequence identity to SEQ ID NO:1, wherein one or more amino acid residues at a position selected from the group consisting of positions 159, 160, 173, 177, 238, 241, 242, 248, 251, 253, 258, 264, 282, 291, 293, 297, 299, 303, 307 and 308, more preferably selected from the group consisting of positions 238, 241, 242, 248, 253, 258, 264, 291, 293, 297, 299, 303, 307 and 308 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence, are substituted with another amino acid residue and wherein said mevalonate diphosphate decarboxylase has improved activity in converting 3-phosphonoxyisovalerate into isobutene.

In one particular embodiment it is preferred that one or more amino acid residues at a position selected from the group consisting of positions 293, 297, 299, 303, 307 and 308 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence, are substituted with another amino acid residue. These residues are located in the most C-terminal part of the enzyme (in alpha helix 9 and

beta strand 12) and might create a more stable confirmation that stabilizes the whole structure.

[0043] A third group of identified positions is located in or close to the binding pocket of the enzyme. This group encompasses positions 16, 23, 24, 28, 31, 111, 139, 141, 142, 182, 186, 188, 279 and 282. The MDP binding pocket is delimited by regions 13-22 and 97-107 that form the ATP binding site and includes in particular the P-loop (P99-S107). Another side consists of the region 274-280 where the catalytic base D276 is located and the region 136-146 which includes R144 that drives the final decarboxylation step of the substrate. Position S105 lies within the phosphate binding loop (consensus GHMP kinase P-Xaa-GLSASAA ->PTAAGLSSSSS in *S.mitis*). Interestingly the mutation that improves the production of IBN is a substitution to Ala that is closer to the consensus P-binding loop sequence compared to the wild type *S.mitis* MDP P-loop sequence. S141 is closely located to the R144 residue and its hydroxyl group interacts with the natural enzyme substrate and is thus believed to be important to determine the enzyme specificity. Substitution of S141 with threonine which has a bulkier side chain could potentially facilitate the interaction of the enzyme with the smaller sized mono-phosphorylated substrate PIV. Substitution to proline would drastically alter the structure of the region and might better accommodate this non-natural substrate PIV. I16, K24R and M28 are neighboring K22 which interacts with the phosphate groups of the natural substrate; altering this residues could affect the structure of this loop and bring K22 closer to the substrate. The best performing mutants harbor the K24R which alters the length of the side chains while increasing the positively charged environment of the binding pocket and the mutation M28K which adds a positive charge. P279 and K282 are close to the catalytic base D276 and R186 is neighboring S185 which interacts with the MVAPP substrate.

Thus, there is also disclosed a variant of a mevalonate diphosphate decarboxylase having an amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 40%, 50%, 60% or 90% sequence identity to SEQ ID NO:1, wherein one or more amino acid residues at a position selected from the group consisting of positions 16, 24, 28, 141, 186, 279 and 282 in the amino acid sequence shown in SEQ ID NO:1 or at a position corresponding to any of these positions in a related sequence, are substituted with another amino acid residue and wherein said mevalonate diphosphate decarboxylase has improved activity in converting 3-phosphonoxyisovalerate into isobutene.

[0044] There is also disclosed a method for providing a variant of a mevalonate diphosphate decarboxylase wherein said variant shows an improved activity of converting 3-phosphonoxyisovalerate into isobutene said method comprising the step of effecting one or more changes in the sequence of the mevalonate diphosphate decarboxylase wherein said change(s) is/are effected at one or more amino acid positions selected from the group consisting of the amino acid positions corresponding to positions 282, 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267, and 315 in the amino acid sequence shown in SEQ ID NO:1. "Corresponding to" means corresponding to any of these positions in a related sequence.

[0045] As regards the preferred embodiments of a mevalonate diphosphate decarboxylase to be mutated according to such a method, the same applies as has been set forth herein-above.

In one preferred embodiment the mevalonate diphosphate decarboxylase from which the variant is derived is a mevalonate diphosphate decarboxylase which shows the amino acid sequence as shown in SEQ ID NO:1 or an amino acid sequence having at least 60% or 90% sequence identity to SEQ ID NO:1 or any of the preferred degrees of sequence identity as specified herein above.

Moreover, as regards preferred embodiments of the degree of improvement in activity and the changes to be effected, the same applies as described herein above.

[0046] The MDP decarboxylase of the present invention can be fused to a homologous or heterologous polypeptide or protein, an enzyme, a substrate or a tag to form a fusion protein. Fusion proteins in accordance with the present invention will have the same improved activity as the MDP decarboxylases of the present invention. Polypeptides, enzymes, substrates or tags that can be added to another protein are known in the art.

They may be useful for purifying or detecting the proteins of the invention. For instance, tags that can be used for detection and/or purification are e.g. FLAG-tag, His6-tag or a Strep-tag. Tags for improving solubility or stability of the enzyme are MBP or ATS. Alternatively, the protein of the invention can be fused to an enzyme e.g. luciferase, for the detection or localisation of said protein. Other fusion partners include, but are not limited to, bacterial β -galactosidase, trpE, Protein A, β -lactamase, alpha amylase, alcohol dehydrogenase or yeast alpha mating factor.

It is also conceivable that the polypeptide, enzyme, substrate or tag is removed from the protein of the invention after e.g. purification.

Fusion proteins can typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods known in art.

[0047] The present invention further relates to a nucleic acid molecule encoding the enzymes, more preferably the MDP decarboxylase variants of the present invention and to a vector comprising said nucleic acid molecules. Vectors that can be used in accordance with the present invention are known in the art. The vectors can further comprise expression control sequences operably linked to the nucleic acid molecules of the present invention contained in the vectors. These expression control sequences may be suited to ensure transcription and synthesis of a translatable RNA in bacteria or fungi. Expression control sequences can for instance be promoters. Promoters for use in connection with the nucleic acid molecules of the present invention may be homologous or heterologous with regard to its origin and/or with regard to the gene to be expressed. Suitable promoters are for instance promoters which lend themselves to constitutive expression. However, promoters which are only activated at a point in time determined by external influences can also be used. Artificial and/or chemically inducible promoters may be used in this context. Preferably, the vector of the present invention is an expression vector. Expression vectors have been widely described in the literature. As a rule, they contain not only a selection marker gene and a replication-origin ensuring replication in the host selected, but also a bacterial or viral promoter, and in most cases a termination signal for transcription. Between the promoter and the termination signal there is in general at least one restriction site or a polylinker which enables the insertion of a coding DNA sequence. The DNA sequence naturally controlling the transcription of the corresponding gene can be used as the promoter sequence, if it is active in the selected host organism. However, this sequence can also be exchanged for other promoter sequences. It is possible to use promoters ensuring constitutive expression of the gene and inducible promoters which permit a deliberate control of the expression of the gene. Bacterial and viral promoter sequences possessing these properties are described in detail in the literature. Regulatory sequences for the expression in microorganisms (for instance *E. coli*, *S. cerevisiae*) are sufficiently described in the literature. Promoters permitting a particularly high expression of a downstream sequence are for instance the T7 promoter (Studier et al., *Methods in Enzymology* 185 (1990), 60-89), lacUV5, trp, trp-lacUV5 (DeBoer et al., in Rodriguez and Chamberlin (Eds), *Promoters, Structure and Function*; Praeger, New York, (1982), 462-481; DeBoer et al., *Proc. Natl. Acad. Sci. USA* (1983), 21-25), lp1, rac (Boros et al., *Gene* 42 (1986), 97-100). Inducible promoters are preferably used for the synthesis of polypeptides. These promoters often lead to higher polypeptide yields than do constitutive promoters. In order to obtain an optimum amount of polypeptide, a two-stage process is often used. First, the host cells are cultured under optimum conditions up to a relatively high cell density. In the second step, transcription is induced depending on the type of promoter used. In this regard, a tac promoter is particularly suitable which can be induced by lactose or IPTG (=isopropyl- β -D-thiogalactopyranoside) (deBoer et al., *Proc. Natl. Acad. Sci. USA* 80 (1983), 21-25). Termination signals for transcription are also described in the literature.

Preferably, the nucleic acid molecules according to the invention are non-naturally occurring nucleic acid molecules, i.e., they are molecules which do not occur in nature. Such non-naturally occurring molecules differ significantly from naturally occurring nucleic acid molecules due to a difference, e.g., in structure. For example, such a non-naturally occurring nucleic acid molecule may encode a non-naturally occurring enzyme as described herein-above. Also a vector according to the present invention is preferably a non-naturally occurring vector, e.g., due to the presence of a non-naturally occurring nucleic acid molecule or due to the combination of elements which do not occur in such a combination in nature.

[0048] In addition, the present invention relates to a host cell comprising the vector of the present invention.

In a preferred embodiment, the host cell according to the presenting invention is a microorganism, in particular a bacterium or a fungus. In a more preferred embodiment, the host cell of the present invention is *E. coli*, a bacterium of the genus *Clostridium* or a yeast cell, such as *S. cerevisiae*. In another preferred embodiment the host cell is a plant cell or a non-human animal cell.

The transformation of the host cell with a vector according to the invention can be carried out by standard methods, as for instance described in Sambrook and Russell (2001), *Molecular Cloning: A Laboratory Manual*, CSH Press, Cold Spring Harbor, NY, USA; *Methods in Yeast Genetics, A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, 1990. The host cell is cultured in nutrient media meeting the requirements of the particular host cell used, in particular in respect of the pH value, temperature, salt concentration, aeration, antibiotics, vitamins, trace elements etc. Such a host cell according to the present invention is preferably a non-naturally occurring host cell, i.e., a host cell which does not occur in nature. Such a non-naturally occurring host cell differs from a naturally occurring cell due to a modification with a nucleic acid molecule or vector as described herein-above.

[0049] The present invention also relates to the use of the MDP decarboxylase of the present invention or a host cell comprising said MDP decarboxylase for the conversion of 3-hydroxyisovalerate or of 3-phosphonoxyisovalerate into isobutene.

[0050] In addition, the present invention relates to a method for producing isobutene from 3-hydroxyisovalerate or from 3-phosphonoxyisovalerate comprising the steps of: Culturing the host cell of the present invention in a suitable medium and recovering said isobutene.

[0051] It has been described previously that the MDP decarboxylase is able to catalyze the conversion of mevalonate into isoprenol via the intermediate mevalonate-3-phosphate (see WO 2011/076261). The inventors could show that also the MDP decarboxylase variants according to the present invention are able to catalyze this conversion and in particular the conversion of mevalonate-3-phosphate into isoprenol. Therefore, the present invention also relates to the use of an MDP decarboxylase variant according to the present invention and as described herein above or of a microorganism expressing such a variant for the conversion of mevalonate or mevalonate-3-phosphate into isoprenol.

[0052] In addition, the present invention relates to a method for producing isoprenol from mevalonate or from mevalonate-3-phosphate comprising the steps of: Culturing the host cell of the present invention in a suitable medium and recovering said isoprenol.

[0053] It has also been described that the MDP decarboxylase is able to catalyze the conversion of 3-hydroxypent-4-enoate into 1,3-butadiene (see PCT/EP2012/075921) via the intermediate 3-phosphonoxypent-4-enoate. The inventors could show that also the MDP decarboxylase variants according to the present invention are able to catalyze this conversion, in particular the conversion of 3-phosphonoxypent-4-enoate into 1,3-butadiene. Therefore, the present invention also relates to the use of an MDP decarboxylase variant according to the present invention and as described herein above or of a microorganism expressing such a variant for the conversion of 3-hydroxypent-4-enoate or of 3-phosphonoxypent-4-enoate into 1,3-butadiene.

[0054] In addition, the present invention relates to a method for producing 1,3-butadiene from 3-hydroxypent-4-enoate or from 3-phosphonoxypent-4-enoate comprising the steps of: Culturing the host cell of the present invention in a suitable medium and recovering said 1,3-butadiene.

[0055] In the above described methods, the microorganisms are cultivated under suitable culture conditions allowing the occurrence of the enzymatic reaction of the MDP decarboxylases of the present invention. The specific culture conditions depend on the specific microorganism employed but are well known to the person skilled in the art. The culture conditions are generally chosen in such a manner that they allow the expression of the genes encoding the MDP decarboxylases of the present invention. Various methods are known to the person skilled in the art in order to improve and fine-tune the expression of certain genes at certain stages of the culture

such as induction of gene expression by chemical inducers or by a temperature shift.

In another embodiment, the above described methods of the invention comprise the step of providing the organism, preferably the microorganism carrying the respective enzyme activity or activities in the form of a (cell) culture, preferably in the form of a liquid cell culture, a subsequent step of cultivating the organism, preferably the microorganism in a fermenter (often also referred to a bioreactor) under suitable conditions allowing the expression of the respective enzyme and further comprising the step of effecting an enzymatic conversion of a method of the invention as described herein above. Suitable fermenter or bioreactor devices and fermentation conditions are known to the person skilled in the art. A bioreactor or a fermenter refers to any manufactured or engineered device or system known in the art that supports a biologically active environment. Thus, a bioreactor or a fermenter may be a vessel in which a chemical/biochemical process like the method of the present invention is carried out which involves organisms, preferably microorganisms and/or biochemically active substances, i.e., the enzyme(s) described above derived from such organisms or organisms harboring the above described enzyme(s). In a bioreactor or a fermenter, this process can either be aerobic or anaerobic. These bioreactors are commonly cylindrical, and may range in size from litres to hundreds of cubic meters, and are often made of stainless steel. In this respect, without being bound by theory, the fermenter or bioreactor may be designed in a way that it is suitable to cultivate the organisms, preferably microorganisms, in, e.g., a batch-culture, feed-batch-culture, perfusion culture or chemostate-culture, all of which are generally known in the art.

The culture medium can be any culture medium suitable for cultivating the respective organism or microorganism.

[0056] The method according to the invention furthermore comprises the step of collecting gaseous products, i.e. isobutene, degassing out of the reaction, i.e. recovering the products which degas, e.g., out of the culture. Thus in a preferred embodiment, the method is carried out in the presence of a system for collecting isobutene under gaseous form during the reaction.

As a matter of fact, short alkenes such as isobutene adopt the gaseous state at room temperature and atmospheric pressure. The method according to the invention therefore does not require extraction of the product from the liquid culture medium, a step which is always very costly when performed at industrial scale. The evacuation and storage of the gaseous hydrocarbons and their possible subsequent physical separation and chemical conversion can be performed according to any method known to one of skill in the art.

[0057] The present invention is further described by reference to the following non-limiting figures and examples.

Figure 1:

General diagram of the directed evolution approach

Figure 2:

General schematic outline of the screening process

Figure 3:

Results obtained by analyzing the quantity of isobutene produced by a subset of the mutants from Table 1 using the activity assay with purified protein

Figure 4:

Panel of substitutions at position K282 and Y121 that improve the activity in converting 3-phosphonoxyisovalerate into isobutene a) Mutational analysis at position K282 indicates that conservative substitutions may have a similar effect on the production of isobutene. i.e.: Substitutions of K282 into nucleophilic S or C or to hydrophobic V and A increase the activity of the enzyme (assay set up using clarified cell lysate - n=3); b) Mutational analysis at position Y121 indicates that conservative substitutions may have a similar effect on the production of isobutene (assay set up using crude cell lysate - n=3).

Figure 5:

The production rate of isobutene (mole of isobutene /mole enzyme/sec) was plotted as a function of the concentration of 3-phosphonoxyisovalerate and the curve was fitted using Michealis Menten equation

$$(V = (V_{\max} * (\text{substrate})) / (K_m + (\text{substrate})))$$

Figure 6:

3D structure of the *S. mitis* MDP decarboxylase (SEQ ID NO:1) and the 3D structure of the MDP decarboxylase from *S. pyogenes* MDD which shows 69% sequence identity to SEQ ID NO: 1. The 3D structure was established by using the MODELER algorithm implemented in the Discovery Studio platform (version 3.0, Accelrys, San Diego, CA). 100 structures were generated and selection was based on minimization of total energy and analysis of the structural quality of the models according to the MODELER probability density functions. The selected structure was used for further studies. *In silico* mutagenesis was performed in Discovery studio 3.0 to visualize the mutations in the 2B4 variant.

Figure 7:

Sequence of the MDP decarboxylase in which the secondary structure (β -sheets and α -helices) as well as the mutated residues are highlighted.

Figure 8:

1. (A) Mevalonate diphosphate decarboxylase structure is characterized by a cone-shaped fold, where the N-terminal region is orientated orthogonally against the relatively planar C-terminal region comprising 5 α -helices. The active site is a positively charged cleft formed between the N and C terminal domains with an ATP binding P loop nearby. Classification of the mutations according to their location in the structure: residues are visualized in yellow.
2. (B) 3-D structure showing the location of residues 9, 11, 42, 43 and 45 on beta strands 1 and 4 that are directly adjacent to each other.
3. (C) Residues in the C-terminal domain: The majority of these residues are situated in a region defined by alpha helices 8-9 and beta strands 9-12. Mutations targeting residues located the most C-terminally L293, F297, Q299, L303, K307 and T308 (alpha helix n°9 and beta-strand n°12) might create a more stable confirmation that stabilizes the whole structure.

Figure 9:

Scheme for the chemical synthesis of 3-phosphonoxyisovalerate.

Figure 10:

Sequence alignment of SEQ ID NO:1 with MDP decarboxylases showing between 60% and 80% sequence identity to SEQ ID NO:1.

Figure 11:

Sequence alignment of SEQ ID NO:1 with MDP decarboxylases showing between 80% and 90% sequence identity to SEQ ID NO:1.

Figure 12:

Sequence alignment of SEQ ID NO:1 with MDP decarboxylases showing between 90% and 100% sequence identity to SEQ ID NO:1.

Figure 13:

Apparent kcat for MDP decarboxylase variants F9, F9-S141P, F9-I16L-R91H-S141P-K241M-S248T-Q299K.

Figure 14:

Conversion of mevalonate-3-phosphate into isoprenol by several mutants.

Figure 15:

shows 1,3-butadiene production from (R)-3-hydroxypent-4-enoate in the following enzymatic assays:

Assay A: without enzyme

Assay B: in the presence of 0.5 mg of MDP decarboxylase *Th. acidophilum* mutant L200E

Assay C: combined assay containing 0.5 mg of mutant L200E of MDP decarboxylase *Th. acidophilum* and 5 mg of mutant 2B4 of MDP decarboxylase *S. mitis*.

Figure 16:

shows 1,3-butadiene production from (R)-3-hydroxypent-4-enoate in the following enzymatic assays:

Assay A: without enzyme

Assay B: in the presence of 0.5 mg of MDP decarboxylase *Th. acidophilum* mutant L200E

Assay C: combined assay containing 0.5 mg of mutant L200E of MDP decarboxylase *Th. acidophilum* and 5 mg of mutant F9 of MDP decarboxylase *S. mitis*.

EXAMPLES

Materials and methods

Methods used to construct and select mutations

a. Directed evolution strategy

[0058] The enzyme *Streptococcus mitis* MDP decarboxylase is capable of catalyzing, amongst other reactions, the reaction of phosphorylation of 3-hydroxyisovalerate into 3-phosphonoxyisovalerate and the reaction of decarboxylation of 3-phosphonoxyisovalerate into isobutene. A directed evolution approach was used in order to specifically improve the rate of conversion of 3-phosphonoxyisovalerate into isobutene by *S. mitis* MDP decarboxylase. This approach consisted in (1) the generation of an initial collection of single point mutants for *S. mitis* MDP decarboxylase, (2) the design of assay systems to test the activity of these enzyme variants, (3) the use of the activity assays to screen the collection of mutants in order to identify mutants with improved activity compared to the activity of the wild type *S. mitis* MDP decarboxylase, (4) additional rounds of evolution (library construction and screening) using as starting material the best mutants identified during the previous round of evolution (see Figure 1). This approach led to the identification and characterization of a collection of mutants with increased activity compared to the wild type enzyme. During the sequential rounds of evolution, a range of molecular biology techniques were used to create the library and mutant collection to be screened. The activity assays consist generally of several steps in order to eliminate false negatives or assay artifacts amongst the initial positive hits and only retain true leads. The activity assays were modified accordingly with the increase in activity rate of the isolated mutants in order to adjust its sensitivity and throughput.

b. Construction of *Streptococcus mitis* MDP decarboxylase enzyme mutants

[0059] The polynucleotide sequences coding for the different mutants identified during the evolution of the *Streptococcus mitis* MDP decarboxylase enzyme were generated using a range of standard molecular biology techniques. All these techniques used a codon-optimised polynucleotide sequence for expression in *Escherichia coli* as template (see SEQ ID NO: 4;). The sequence optimization has been done by Geneart using their GeneOptimizer software.

Different PCR-based techniques known in the art were used for the construction of single point mutants. For the generation of enzyme variants bearing multiple mutations (at least two mutations), either PCR-based techniques or other methods known in the art were used to introduce these mutations. Following mutagenesis, the mutated polynucleotide sequence was inserted into an expression vector (used for recombinant protein production in *E. coli* and screening) either using standard ligase-based subcloning techniques, whole plasmid extension by PCR or ligase-independent cloning techniques (LIC; Life Technology Gateway® recombinant technology).

c. Selection of the enzyme mutants with increased activity

Description of the screening method (see Figure 2):

[0060] The DNA library or the collection of mutants inserted into the expression vector was transformed into the commercially available recombinant protein expression *E. coli* strain BL21DE3. The DNA library transformation reaction was plated out onto LB Ampicillin plates and isolated clones were used to inoculate a starter culture. When transforming a collection of mutants, each clone was individually transformed, plated out and an isolated clone used to inoculate a starter culture; or the transformation mixture for each individual clone was directly used to inoculate a starter culture. The protein expression was carried out using an auto-induction medium (ZYM-5250) as described in Studier F.W (Protein Expr. Purif. 41 (2005), 207-234) with an initial cell growth phase for 6 hours at 37°C and an induction stage overnight at 28°C. After the transformation, all subsequent steps were carried out in microplates or deepwell plates. The cell cultures were centrifuged and the pellets were stored for at least 1 hour at - 80°C. The cell pellets were then resuspended in a small volume of buffer and lysed by sonication before being subjected to centrifugation for the removal of cell debris.

Screening for variants with an increased activity of converting 3-phosphonoxyisovalerate into isobutene

[0061] To test the activity of the mutant enzyme to catalyze the conversion of 3-phosphonoxyisovalerate into isobutene, a reaction mix was prepared in glass GC vials by mixing cell lysates (supernatant), 3-phosphonoxyisovalerate substrate in 50 mM Tris pH 7 final concentration. 3-phosphonoxyisovalerate has been prepared enzymatically by incubating 3-hydroxyisovalerate, purified *Thermoplasma acidophilum* MDP decarboxylase and cofactors (ATP, MgCl₂, KCl) at 37°C for 24 hours. The reaction mixture was incubated for varying length of time at 37°C and the gas phase was injected in a gas chromatograph alongside the appropriate references (isobutene for calibration, wild type enzymes, negative controls...). Following analysis of the GC chromatograms, mutants showing an activity increased by at least 20% were selected and subjected to a second round of screening which followed the same conditions as the primary screen.

[0062] The final step in the selection process included the production and purification of the top hits, i.e. enzyme variants with the highest improved activity validated by the primary and secondary screens and their activity was tested with different substrate concentrations. Confirmed hits were further characterized and the kcat and Km for the reaction was calculated.

Modification of the screening process:

[0063] As mentioned before, the screening protocol was regularly modified as new improved mutants were identified. The main modification of the protocols covers the following points:

- Cell culture conditions: mode of inoculation (using isolated clone or transformation reaction), medium volume (200 µl to 5 ml), 24 deepwell plates, 96 deepwell and standard 96-well microplates, agitation varies according to the type of shaker incubator
- Cell lysis: type and volume of buffer used, sonication or no sonication, centrifugation or not (crude lysate can be used effectively)
- Set up of the enzymatic reaction:
 - Either 3-phosphonoxyisovalerate is enzymatically prepared prior to the assay (concentration of enzyme *T. acidophilum*, cofactors, HIV ... variable as well as the incubation time and temperature)
 - The reaction of phosphorylation of 3-hydroxyisovalerate and the decarboxylation of 3-phosphonoxyisovalerate into isobutene is combined in one tube: *S. mitis* MDD mutant is combined with purified *T. acidophilum* and 3-hydroxyisovalerate. The reaction is incubated and assayed for the presence and quantity of isobutene.
 - Pure 3-phosphonoxyisovalerate compound chemically synthesized is used in the assay. 3-

phosphonoxyisovalerate (PIV) was chemically synthesized from 3-hydroxyisovalerate according to the scheme depicted in Figure 9 by SYNTHEVAL (France)

- Subjecting the enzymatic reaction to agitation during incubation leads to a 5 fold increase in signal
- Number of steps in the screening can vary
- Number of replicates analyzed for each clone vary (1 reaction per clone in the primary screens, clone can be tested in duplicate, triplicate or more in the following screens)
- Method of GC analysis can vary: type of columns, type of vials and septa, and method (oven, injectors, detectors, temperature, time of analysis...)

Example 1: Identification of single point mutants of *S. mitis* MDP decarboxylase with increased activity for the reaction of conversion of 3-phosphonoxyisovalerate into isobutene

[0064] A collection of 2632 single point mutants of *S. mitis* MDP decarboxylase was prepared using standard molecular biology techniques. Saturation mutagenesis aimed at substituting systematically all the 317 amino acids of *S. mitis* MDP decarboxylase with the 19 non wild type amino acids. The average number of substitutions per position was 8 out of the 19 possible. The coding sequence for each individual mutant was sub-cloned in an expression vector to allow the production of an N-terminal 6-His-tagged recombinant mutant enzyme in *E. coli*.

[0065] The recombinant protein expression *E. coli* strain BL21 DE3 was transformed with the expression vectors encoding the mutant enzymes, the empty expression vector (negative control) and the expression vector encoding the wild type enzyme (positive control). To speed up the process, the transformation was carried out in a 96-well plate. Briefly, 2 µl of DNA plasmid miniprep of each mutant and each control was transferred per well into a 96-well 0.2 ml PCR reaction plate before addition of 40 µl/well of chemically competent BL21 DE3 *E. coli* cells. The plate was incubated on ice for 15 minutes before carrying out a heatshock for 1 minute at 42°C in the AB 2720 Thermal cycler. The plate was then immediately placed on ice and cooled for 1 minute before 1 ml of sterile Luria-Bertani medium was added (10 g/l Tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7). The plate was sealed using gas-permeable adhesive film and incubated for 45 minutes at 37°C at 200 rpm in an Infors Minitrion orbital shaker. 50 µl of the transformation mixture was then used to inoculate 0.5 ml of Luria-Bertani medium supplemented with 100 µg/ml ampicillin placed in a 96 deepwell plate. These plates were sealed and incubated overnight at 37°C at 200 rpm. Bacterial stocks were prepared in a 96-well plate by mixing 100 µl of the overnight starter cultures with 35 µl sterile 50% glycerol and stored at -80°C until further use.

[0066] For the production of the recombinant mutant enzyme, 1 ml of sterile autoinduction medium (Studier F.W, Protein Expr. Purif. 41 (2005), 207-234) supplemented with the appropriate antibiotic, was distributed in the wells of a 96-deep well plate and said wells were inoculated with 10 µl of thawed glycerol stocks. Each plate contained approximately 70 to 80 distinct mutant enzymes, 8 negative controls (empty expression vector) and 8 wild type enzyme clones for use as a reference. Plates were incubated for 6 hours at 37°C at 1000 rpm in a Heidolph Titramax orbital shaker followed by a further overnight incubation at 28°C at 1000 rpm. Bacterial cells were pelleted by centrifugation for 20 minutes at 3200 x g at 4°C. Cell pellets were stored at -80°C.

[0067] Pellets were thawed on ice for 5 to 10 minutes and were resuspended in 250 µl of resuspension buffer (50 mM Tris-Cl pH7, 20 mM KCl, 10 mM MgCl₂, 10% glucose, 1 µl/ml Merck-Novagen Lysonase). Cell suspensions were incubated at room temperature for 15 minutes and on ice for 30 minutes. Bacterial cells lysis was carried out by sonicating these cell suspensions for 4 pulses of 5 minutes in an Advantage Lab ultrasonic water bath filled with ice and water (5 minutes of rest on ice between pulses). Cell lysates were then centrifuged for 20 minutes at 10°C at 3200 x g to pellet cell debris and 240 µl of the supernatants were transferred into a fresh plate. The enzymatic reaction was set up in Agilent 2 ml glass vials by mixing 200 µl of the supernatant with 300 µl of 3-phosphonoxyisovalerate substrate. The vials were hermetically sealed using crimp caps (PTFE-

silicon-PTFE coated), incubated in a waterbath for 24 hours at 37°C and stored at -20°C prior to analysis by gas chromatography. To prepare 3-phosphonoxyisovalerate substrate, 0.063 mg/ml of purified *Thermophilus acidophilum* MDP decarboxylase was mixed with 50 mM hydroxyisovalerate, 40 mM Adenosine tri-phosphate in a 50 mM Tris-Cl pH7, 20 mM KCl, 20 mM MgCl₂ and incubated for 24 hours at 45°C. This enzymatically prepared 3-phosphonoxyisovalerate substrate was aliquoted and stored at -20°C until further use.

[0068] The isobutene produced by the enzymatic reaction in the presence of *S. mitis* MDP decarboxylase was quantified by gas chromatography. The vials were thawed quickly at 30°C for 30 minutes and placed on an automated sampler mounted onto a Varian GC-430 system equipped with a Varian CP SilicaPlot column (30 m x 0.32 mm), one injection port and one Flame Ionization detector (FID). The sampler was set up to inject 100 µl of headspace gas. For the GC analysis method used to detect isobutene, oven temperature was set to 185°C, injector port temperature was set to 150 °C with a split ratio of 4:1 and the FID detector to 250°C. The GC uses nitrogen as carrier gas (constant flow of 1.5 ml/min) and a mixture of air (air flow 28 ml/min) and hydrogen (300 ml/min) for the FID detection system. The duration of the analysis was approx. 3 minutes per sample and under these conditions isobutene was observed to elute at 2.5 min. A sample of commercially bought pure isobutene was injected prior to the start of the analysis to calibrate the GC system and to determine the retention time for isobutene. Following the analysis, chromatograms were processed using Galaxy software; the area under the peak was integrated for each mutant and compared to that of the wild type enzyme. Mutants showing an increase of at least 10-15% of isobutene production compared to the amount produced by the wild type enzyme were tested for a second time according to the protocol described above in order to eliminate false positives. Finally all mutant enzymes that have been selected through these two rounds of screening were tested once again using normalized quantities of purified protein in the assay. Briefly, the bacterial glycerol stocks of the selected mutant enzymes were used to inoculate 7 ml of LB-Amp. 2 ml of this starter culture was used to inoculate 200 ml of autoinduction medium and the plasmid DNA was extracted from the 5 ml of culture leftover. The plasmid DNA was sequenced to confirm the presence and the type of a mutation. The bacterial expression for the production of the mutant enzymes was carried out as previously described and the N-terminal 6His-tagged mutant enzymes were purified from the pellet using Macherey-Nagel Protino purification kit according the users' manual. The activity was determined by mixing in a 2 ml GC vial: 500 µg of purified enzyme, 300 µl of 3-phosphonoxyisovalerate substrate and the volume was adjusted to 500 µl with 50 mM Tris-Cl pH7. The reaction was incubated for 24 hours at 37°C and stopped by freezing the samples at -20°C. The quantity of isobutene produced was determined by GC analysis.

[0069] This screening procedure led to the identification of 71 mutations which confer *S. mitis* MDP decarboxylase an increased activity of isobutene production. Table 4 lists the position and type of substitutions that have been identified. Figure 3 shows the results obtained by analyzing the quantity of isobutene produced by a subset of the mutants from Table 4 using the activity assay with purified protein. Additional data is represented by Figures 4 a) and b).

QY121	K123	S129	E134	% increase compared to wild type enzyme
				<300%
				<300%
				>100%
				75-100%
				50-75
	M			25-50%
W		P.V	G	10-25%

QZ99	L303	K307	T308	% increase compared to wild type enzyme
				<300%
				<300%
				>100%
				75-100%
				50-75
	M			25-50%
P		H	S	10-25%

Table 4: List of the position and type of substitutions t

[illegible]

Example 2: Characterization of variants of *S. mitis* MDP decarboxylase with high turnover rate

[0070] A collection of *S. mitis* MDP decarboxylase variants has been created by combining a selection of single point mutations which confer high increase in activity (increase of at least 50% compared with the activity of the wild type enzyme) using PCR-based techniques. Additional diversity was also created by random mutagenesis. The quantity of isobutene produced by these variants was determined in an enzymatic assay whereby 500 µg of purified enzyme was mixed to 300 µl of 3-phosphonoisovalerate substrate in 50 mM Tris-Cl pH7 buffer. Following a 24-hour incubation period at 37°C, the reaction was stopped by freezing the samples at -20°C and the quantity of isobutene produced was determined by GC analysis. For the GC headspace assay, 100 µl of the headspace gas was injected in the injector port of a Varian GC-430 system equipped with a Varian CP SilicaPlot column (30 m x 0.32 mm) and a FID. The GC analysis method used to detect isobutene is characterized by an oven temperature at 185°C, injector port temperature at 150°C with a split ratio of 1:10 and the FID detector temperature at 250°C. Nitrogen was used as carrier gas (constant flow of 1.5 ml/min) and a mixture of air (air flow 28 ml/min) and hydrogen (300 ml/min) were used to supply the FID detection system.

A number of variants showing mutations of up to 11 positions have been identified which show an increased activity in the activity assay. The different variants are shown in the following Tables.

Table 5: Double mutants

Mutations	% increase in activity compared to wild type enzyme
K282CM42A	483
K282CL264Q	453
K282CL303M	510
K282CC118L	465
K282CA253i	493
K282CA 120N	507
K282CE45M	516
K282CF297L	471

Mutations	% increase in activity compared to wild type enzyme
K282CY11 C	508
K282CY121R	400
K282CS 129V	514
K282CA120L	435
K282CK116L	440
K282R9L	428
K282CY11E	270
K282CE177V	493
K282CK116R	479
K282CA120I	205
K282CK116M	576
K282CT251M	452
K282CL293F	375
K282CK123M	178
K282CF122M	233
K282CY121L	290

Table 6: Triple mutants

Mutations	% increase in activity compared to wild type enzyme
E45LY121RK282C	420
K282CY121RY11E	230
K116IY121RK282C	147
Y121RE177VK282C	524
A120RY121LK282C	184
M173CK282CF297L	500

Table 7: Combinations of 4 mutations

Mutations	% increase in activity compared to wild type enzyme
Y121 RK282CL303MT308S	377
M173CL303MK307HT308S	480
E45VM173CK282CL303M	500

Table 8: Combinations of 5 mutations

Mutations	% increase in activity compared to wild type enzyme
Y121 RM173CK282CL303MT308S	507
E159LM173CL303MK307HT308S	480
R9LY11FL303MK307HT308S	440
C118LY121 RM173CK282CL303M	500

Table 9: Combinations of 6 mutations

Mutations	% increase in activity compared to wild type enzyme
Y121 RE159LM173CK282CL303MT308S	558
Y121 RE159LM173CV215AK282CL303M	500
E45LE159LM173CK282CF297LT308S	550

Table 10: Combinations of 7 mutations

Mutations	% increase in activity compared to wild type enzyme
Y121RE159LM173CK282CL303MK307HT308S	643
E45VY121RE159LM173CK282CL303MT308S	500
Y121RE159LM173CK282CF297LL303MT308S	500
C118LY121RE159LM173CK282CL303MT308S	500
Y121RE159LM173CE177CK282CL303MT308S	500
Y121RE159LM173CT242AK282CL303MT308S	500
C118LY121RE159LM173CK282CL303MG315S	500
C118WY121 RE159LM173CE177CK282CL303M	500
C118LY121 RE159LM173CE177CK282CL303M	500
E45VY121RM173CK282CF297LL303MT308S	500
E45VC118LY121RM173CK282CL303MT308S	550
E45VY121RE159LM173CK282CF297LL303M	550

Table 11: Combinations of 8 mutations

Mutations	% increase in activity compared to wild type enzyme
D80GY121 RE159LM173CK282CL303MT308SG315S	500
K24RY121 RE 159LK 123RM173CM258LK282CL303M	500
C118LY121RE159LM173CE177CV215AK282CL303M	500
K24RC118LY121RE159LM173CE177CK282CL303M	500
E45VD80GY121RM173CK282CF297LL303MT308S	500
E45LY121RE159LM173CE177CK282CL303MT308S	550

Table 12: Combinations of 9 mutations

Mutations	% increase in activity compared to wild type enzyme
C118LY121RE159LM173CE177CK282CF297LL303MT308S	500
C118LY121RE159LM173CE177CV215AK282CF297LL303M	500
D80GC118LY121RE159LM173CE177CK282CL303MG315S	500
K24RE45VY121RK123RM173CK282CF297LL303MT308S	550
E45VY121RE159LM173CE177CV215AM258LK282CL303M	550

Table 13: Combinations of 10 mutations

Mutations	% increase in activity compared to wild type enzyme
E45VC118LY121 RE159LM173CV215AK282CF297LL303MT308S	500

Table 14: Combinations of 11 mutations

Mutations	% increase in activity compared to wild type enzyme
K24RC118L Y121 RE159LM173CE177CK282CE291DF297LL303MT308S	750

[0071] Two variants named 2B4 (SEQ ID NO:2) and F9 (SEQ ID NO:3) as having high increase in activity in the assay were selected for further characterization. The 2B4 and F9 protein sequence contain 6 and 11 mutations respectively compared to the wild type enzyme (see Table 15). F9 carries two novel mutations, K24R and E291D that were not identified in the original single point mutations screen.

Table 15: Mutations of variants 2B4 and F9

ID	Mutations	total mutation
2B4	Y121RE159LM173CK282CL303MT308S	6
F9	K24RC118LY121RE159LM173CE177CK282CE291DF297LL303MT308S	11

[0072] Michaelis Menten k_{cat} and K_m steady-state kinetics constants were determined for these two variants as follows: a series of enzymatic reactions were set up in GC vials with 200 μ g of purified 2B4, F9 or the wild type enzyme, a range of 0 to 320 mM of chemically synthesized 3-phosphonooxyisovalerate, 5mM ATP, 20mM KCl, 10mM $MgCl_2$ and 50 mM Tris-Cl pH 7.5. The vials were sealed and incubated for 15 hours at 37°C before analysing the isobutene produced by GC as previously described. Previous experiments had determined that the rate of isobutene production in this experimental set up was constant in the 20 first hours of the enzymatic reaction and thus the rate of isobutene produced per hour as determined after 15-hour incubation is equal to the initial rate of isobutene production at the beginning of the reaction. In order to quantify the absolute amount of isobutene produced by the reaction, the GC was calibrated using a range of concentration of pure isobutene (0 to 10,000ppm). The calibration table was found to be linear in this range of isobutene concentration. The production rate of isobutene (mole of isobutene /mole enzyme/sec) were plotted as a function of the concentration of 3-phosphonooxyisovalerate and the curve was fitted using Michealis Menten equation ($V = (V_{max} * (substrate)) / (K_m + (substrate))$) (Figure 2) to extract the k_{cat} (s-1) and K_m values (mM) that are summarized in Table 16. As shown in Figure 5 and Table 16, variants 2B4 and F9 had a higher k_{cat} than the wild type enzyme.

Table 16: Summary of the k_{cat} (s-1) and K_m values (mM) of the variants 2B4 and F9

ID	K_m (mM)	k_{cat} (10-3.s-1)	k_{cat}/K_m (mM-1.s-1)
wild type enzyme	12.5	2.2	0.18
2B4	34.4	12	0.35
F9	38.38	16	0.42

Example 3: Identification of variants of *S. mitis* MDP decarboxylase with further increased activity for the reaction of conversion of 3-phosphonooxyisovalerate into isobutene

[0073] Additional MVD variants with a further enhanced activity in converting 3-phosphonooxyisovalerate into isobutene were identified through successive rounds of mutagenesis, recombination of point mutations and *in vitro* and/or *in vivo* screening assay. The list of these MVD variants is presented in the following Table 17.

Table 17

Mutations added to F9 variant	Fold Increase	Screening Assay
S141P	3	IN VITRO

Mutations added to F9 variant	Fold Increase	Screening Assay
S141T	2	IN VITRO
S105A	1.8-2	IN VITRO
Q299K	1.25	IN VITRO
I16L	1.25	IN VITRO
S248T	1.4	IN VITRO
K241 M	1.25	IN VITRO
I16L S105A	2.2	IN VITRO
S141P K241M S248T	3.1	IN VITRO
I16L R91H S141P K241M S248T	3.5	IN VITRO
I16L S141P	3.5	IN VITRO
I16L R91H S141P K241M S248T Q299K	3.6	IN VITRO
I16L S141P K241M	3.6	IN VITRO
I16L S141P K241M S248T	3.7	IN VITRO
I16L R91H S141P K241M S248T Q299K M28K	7.24	IN VITRO
I16L R91H S141P K241M S248T Q299K M28A	5.48	IN VITRO
I16L R91H S141P K241M S248T Q299K K180P	5.00	IN VITRO
I16L S141P K241I S248T	5.25	IN VIVO
I16L S141P K241I S248T M28K	8.37	IN VIVO
I16L S141P K241I S248T M28K K180P	11.55	IN VIVO
I16L S141P K241I S248T M28K T53V K180P	11.97	IN VIVO
I16L S141P K241I S248T M28K K180P A238K	15.015	IN VIVO
I16L S141P K241I S248T M28K K180P A238R	16.17	IN VIVO
I16L S141P K241I S248T M28K K180P C282V	15.015	IN VIVO
I16L S141P K241I S248T M28K K180P P279A	17.325	IN VIVO
I16L S141P K241I S248T M28K K180P Y161R	16.17	IN VIVO

[0074] The fold increase is the ratio of MDP decarboxylase variant activity over wild type MDP decarboxylase activity. The fold increase is determined for one substrate concentration (1 or 2 mM PIV for *in vitro* assay and 500mM acetone for *in vivo* assay). The quantity of the enzyme is not normalised but these MVD variants are expressed in similar amounts as observed on SDS-PAGE analysis of cell lysates.

[0075] The template for mutagenesis was the variant named "F9" which corresponds to the *S.mitis* MDP decarboxylase of SEQ ID NO:1 with the following mutations: K24RC118LY121RE159LM173CE177CK282CE291DF297LL303MT308S (see SEQ ID NO: 3). The *in vitro* screening assay used was the one described herein-above section c of Materials and Methods, preferably an *in vitro* assay involving the use of cell lysate.

For the *in vivo* testing a further *in vivo* assay was developed. This assay is based on the use of a bacterial strain transformed with an expression vector that contains the coding sequences and lead to the production of the 3 enzymes involved in the metabolic pathway converting acetone to isobutene; namely for the production of 3-hydroxyisovalerate (HIV) the *M. musculus* HMG-CoA synthase (referred to in the following as HIV synthase) was used; for the phosphorylation of 3-hydroxyisovalerate into 3-phosphonoxyisovalerate (PIV) the *T. acidophilum* MDP decarboxylase was used (referred to in the following as HIV phosphorylase) and for the conversion of PIV into isobutene (IBN) by decarboxylation the *S.mitis* MDP decarboxylase variant to be tested was used (referred to in the following as PIV decarboxylase). This strain is first cultured, overnight in a shaking incubator at

30°C/1000rpm, in 1ml of auto-induction culture medium to produce the three types of recombinant enzymes. The cell pellet containing these three overexpressed recombinant enzymes is then resuspended in 500µl of minimum medium supplemented with 500mM acetone and incubated for a further 16 hours in a shaking incubator set at 37°C/1000rpm. During this second step, HIV synthase catalyses the condensation of acetone (from the medium) with the cellular acetyl CoA into HIV which is then sequentially converted into PIV and IBN by the HIV phosphorylase and the PIV decarboxylase using the cellular ATP. The IBN produced is then quantified by gas chromatography using the same method as the *in vitro* screening assay described in section c of Materials and Methods.

The main advantages of this assay are as follows: (1) the production of IBN occurs within the cell, (2) cellular metabolites (ATP and acetylCoA) and cellular co-factors are used, only acetone is added to the reaction (acetone being a none limiting factor in this pathway due to the efficiency of the clostridial acetone pathway) (3) the increase in IBN production is measured in the context of the complete IBN production pathway and takes into account not only the cooperation of the three enzymes of interests, but also the presence of potential endogenous inhibitors or competitors and low substrate concentrations, (4) enzymes are in their most native form and are not subjected to any form of extraction or a purification process that could denature them and negatively affect their activity.

[0076] For some above described variants, the fold increase in activity has also been determined over a range of substrate concentrations in order to calculate kinetic parameters (apparent k_{cat} and K_m) using equal quantities of MDP decarboxylase variants. The fold increase for the apparent k_{cat} is generally similar to that determined in the screening assays (see Figure 13). In Figure 13, the fold increase for F9-S141P k_{cat} is approx. x3.5 of the F9 k_{cat} compared to x3 determined in the screening assay; the fold increase for F9- I16L-R91H-S141P-K241M-S248T-Q299K k_{cat} is approx. x5.5 of the F9 k_{cat} compared to x3.7 determined in the screening assay.

Example 4: Variants of *S. mitis* MDP decarboxylase also show an increased activity in catalyzing the conversion of mevalonate-3-phosphate into isoprenol

[0077] The ability of MDP decarboxylases variants to convert mevalonate-3-phosphate into isoprenol was evaluated using a coupled-enzymatic assay. This assay combines the sequential activities of two MDP decarboxylases, (1) *T. acidophilum* MDP decarboxylase (L200E mutant) catalyses the phosphorylation of mevalonate into mevalonate-3-phosphate; (2) *S. mitis* MDP decarboxylase catalyses the conversion of mevalonate-3-phosphate into isoprenol. This assay was set up in 50mM Tris-HCl pH7.5, 10mM MgCl₂, 20mM KCl, 40mM ATP using 200mM R,S-sodium mevalonate substrate, 2mg/ml of *S. mitis* MDP decarboxylase variants and 0.1mg/ml of *T. acidophilum* MDP decarboxylase (L200E mutant). Negative controls were also prepared without enzymes or with either enzyme. The reaction mixture was incubated in a sealed glass vial for 24 hours at 37°C. Isoprenol was extracted by mixing 50µl of the reaction mix to 100µl of ethyl acetate. 100µl of the upper ethyl acetate phase was transferred to a clean vial and analysed by Gas chromatography. Commercial isoprenol was used as a reference. The samples were analysed on a Varian GC-430 gas chromatograph equipped with a flame ionization detector (FID). A 1µl sample was analysed on a DB-WAX column (30m, 0.32x0.50µm, Agilent) using the following temperature gradient: 2 minutes at 60°C, temperature ramp to 220°C (20°C/min) and finally 10 minutes at 220°C. In these conditions the retention time of isoprenol was 7.38min.

[0078] It was observed that mutations I160N, R186H, R91H also confer an increase in the activity of *S. mitis* MDP decarboxylase to convert mevalonate-3-phosphate into isoprenol (see Figure 14). The increase of activity is specific to these particular mutations as an increase in the production was detected on either the "2B4" or "F9" variant template.

Example 5: Butadiene production from 3-hydroxypent-4-enoate catalyzed by mutant 2B4 of MDP decarboxylase from *S. mitis*

[0079] The mutant 2B4 is described in Example 2.

(R)-3-hydroxypent-4-enoic acid was synthesized upon request by a company specialized in custom synthesis (Syntheval, France)

The enzymatic reactions were performed under the following conditions:

50 mM Tris-HCl pH 7.5

0-200 mM (R)-3-hydroxypent-4-enoic acid ("R" HPA)

50 mM ATP

20 mM MgCl₂

20 mM KCl

The pH was adjusted to 7.5

Each assay was started by the addition of a particular purified enzyme to 0.5 ml of reaction mixture. The assays were then incubated with shaking at 37°C in a 2 ml sealed vial (Interchim). Control reactions were run in parallel. After 20 hours of incubation the butadiene production was analyzed as follows. One ml of the gaseous phase of each assay was collected and directly injected into a Gas Chromatograph GC-450 (Brucker) equipped with a Flame Ionization Detector (FID). Nitrogen was used as carrier gas with a flow rate of 1.5 ml/min. Volatile compounds were chromatographically separated on Rt-Alumina Bond/Na₂SO₄ column (30 m, 0.32 mm ID, 5 µm) (Restek) using an isothermal mode at 130°C. The enzymatic reaction product was identified by comparison with 1,3-butadiene standard (Sigma). Under these GC conditions, the retention time for butadiene was 7.4 min.

The results are shown in Figure 15: No formation of 1,3-butadiene was observed without substrate. The GC analysis of reactions without enzyme showed only traces of butadiene resulted from the thermal decomposition of the 3-hydroxypent-4-enoate. The catalytic tests showed a significant increase of butadiene production in the presence of mutant 2B4 of MDP decarboxylase from *S. mitis*.

Example 6: Butadiene production from 3-hydroxypent-4-enoate catalyzed by mutant F9 of MDP decarboxylase from *S. mitis*

[0080] The mutant F9 is described in Example 2.

[0081] The enzymatic reactions were performed under the following conditions:

50 mM Tris-HCl pH 7.5

0-200 mM "R" 3-hydroxypent-4-enoic acid ("R" HPA)

50 mM ATP

20 mM MgCl₂

20 mM KCl

The pH was adjusted to 7.5

Each assay was started by the addition of a particular enzyme to 0.5 ml of reaction mixture. The assays were then incubated with shaking at 37°C in a 2 ml sealed vial (Interchim). Control reactions were run in parallel. After 20 hours of incubation the butadiene production was analyzed according to the procedure described in Example 5.

The results are shown in Figure 16: No formation of 1,3-butadiene was observed without substrate. The GC analysis of reactions without enzyme showed only traces of butadiene resulted from the thermal decomposition

of the 3-hydroxypent-4-enoate. The catalytic tests showed a significant increase of butadiene production in the presence of mutant F9 of MDP decarboxylase from *S. mitis*.

Example 7: identification of variants of *S. mitis* MDP decarboxylase with further increased activity for the reaction of conversion of 3-phosphonoxyisovalerate into isobutene

[0082] Additional MVD variants with a further enhanced activity in converting 3-phosphonoxyisovalerate into isobutene were identified through successive rounds of directed or random mutagenesis, recombination of points mutations and in vitro and/or in vivo screening assay. The list of these MVD variants is presented in the following Table 18.

Table 18

Mutations added to F9 variant	Fold Increase	Screening Assay
D2H	1.05	IN VIVO
M42L-D87E-S139C-R186L-K231Q	1.31	IN VIVO
E164Q-R186V-D252E	2.15	IN VIVO
D87E-S139C-R186L-K231Q	2.27	IN VIVO
R186V-Q267R	2.29	IN VIVO
S139C-R186I	2.39	IN VIVO
L111M-F122Y-R186L	2.50	IN VIVO
M751-R186V	2.57	IN VIVO
S139A-S141C	2.60	IN VIVO
K179K-R186V	2.77	IN VIVO
R186V	2.78	IN VIVO
A57S-A58T-K77R-R186V	2.86	IN VIVO
L111M-R186L	2.94	IN VIVO
R186L	2.94	IN VIVO
A31S-R186V	3.03	IN VIVO
S139A-S141G	3.04	IN VIVO
M751-R186L-S308T	3.34	IN VIVO
R186L-S308T	3.40	IN VIVO
R186I	3.46	IN VIVO
L111M-R186V-S308T	3.58	IN VIVO
R186V-D221E	3.67	IN VIVO
L111M-R186V	3.77	IN VIVO
M1L-L111M-R186V-S308T	3.77	IN VIVO
R186N	4.16	IN VIVO
R24S-G86Q-R186I	4.30	IN VIVO
I16L-S141P-K241I-K180P-E227K	20.49	IN VITRO
I16L-S141P-K241I-K180P-D291E-M303L	22.84	IN VITRO
S141 P-K241I-S248T-K180P-R24K	24.46	IN VITRO
I16L-S141 P-K241 I-S248T-L297F	25.01	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-A246E	28.17	IN VITRO

Mutations added to F9 variant	Fold Increase	Screening Assay
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-T242E	29.87	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-Y255E	31.23	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-T198D	31.56	IN VITRO
I16L-S141 P-K241 I-S248T-M28K-K180P-L188C-L297F-K23L	32.24	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-K179L	32.58	IN VITRO
I16L-S141 P-K241 I-S248T-M28K-K180P-L188C-L297F-K231L	32.58	IN VITRO
I16L-S141 P-K241 I-S248T-M28K-K180P-L188C-L297F-P182E	32.58	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-A238E	32.92	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-K208L	32.92	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-R204L	32.92	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-R24L	32.92	IN VITRO
I16L-S141 P-K241 I-S248T-M28K-K180P-L188C-L297F	33.94	IN VITRO
I16L-S141P-K241I-K180P-D291E	35.32	IN VITRO
I16L-S141P-K241I-S248T-K180P-Q267R-R24K-L118C-L159E	39.40	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-S142A	44.78	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L159E-D291E	44.94	IN VITRO
I16L-S141P-K241I-S248T-K180P-G166S-R24K	51.64	IN VITRO
I16L-S141P-K241I-S248T-K180P-R24K	57.93	IN VITRO
I16L-S141P-K241I-S248T-M28K-K180P-L188C-L297F-Q205H	67.88	IN VITRO

[0083] The fold increase is the ratio of MDP decarboxylase variant activity over the "F9" variant MDP decarboxylase activity. The "F9" variant corresponds to the *S. mitis* MDP decarboxylase of SEQ ID NO:1 with the following mutations: K24R-C118L-Y121R-E159L-M173C-E177C-K282C-E291D-F297L-L303M-T308S (see SEQ ID NO:3). The fold increase is determined for one substrate concentration (2 or 6 mM PIV in the *in vitro* assay and 10 mM HIV in the *in vivo* assay). The quantity of the enzyme is not normalized but these MVD variants are expressed in similar amounts as observed on SDS-PAGE analysis of cell lysates.

[0084] The *in vitro* screening assay used in present Example 7 is described herein-above in section c of Materials and Methods, wherein, preferably, an *in vitro* assay involving cell lysates has been used, while the assay has preferably been further miniaturized in 384 deepwell microplates.

[0085] For the *in vivo* testing a further *in vivo* screening assay has been developed as outlined in the following. This assay is based on the use of a bacterial strain transformed with an expression vector containing the coding sequences and leading to the production of the last two enzymes involved in the metabolic pathway converting

acetone to isobutene. More specifically, for the production of 3-phosphonoxyisovalerate (PIV) from the 3-hydroxyisovalerate (HIV), the *T. acidophilum* MDP decarboxylase was used (referred to in the following as HIV phosphorylase) and, for the conversion of PIV into isobutene (IBN), the *S. mitis* MDP decarboxylase variant to be tested was used (referred to in the following as PIV decarboxylase). This strain is first cultured for 24 hours in a shaking incubator at 30°C, 700 rpm, in 300 µL of auto-induction culture medium in 384 deepwell microplates, in order to produce the two types of recombinant enzymes. The cell pellet containing these two overexpressed recombinant enzymes was then resuspended in 50 µL of minimum medium supplemented with 10 mM HIV and incubated further for another four hours in a shaking incubator at 30°C, 700 rpm. During this step, HIV phosphorylase catalyses the phosphorylation of HIV with cellular ATP into PIV, which is then converted into IBN by the PIV decarboxylase variants. The IBN produced is then quantified by gas chromatography using the same method as the *in vitro* screening assay described above in section c of Materials and Methods.

SEQUENCE LISTING

[0086]

<110> Global Bioenergies

Scientist of Fortune S.A.

<120> Mevalonate diphosphate decarboxylase variants

<130> T2557 PCT S3

<150> EP 13 17 5790.8

<151> 2013-07-09

<160> 4

<170> BiSSAP 1.2

<210> 1

<211> 317

<212> PRT

<213> Streptococcus mitis B6

<400> 1

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Ser Ser Ile Ser Leu Thr Leu Glu Asn Met Tyr Thr Glu Thr Thr Leu
35     40     45
Ser Ser Leu Pro Thr Asp Ala Thr Ala Asp Ala Phe Tyr Ile Asn Gly
50     55     60
Gln Leu Gln Asn Glu Ala Glu His Val Lys Met Ser Lys Ile Ile Asp
65     70     75     80
Arg Tyr Arg Pro Asp Gly Asp Gly Phe Val Arg Ile Asp Thr Gln Asn
85     90     95
Ser Met Pro Thr Ala Ala Gly Leu Ser Ser Ser Ser Ser Gly Leu Ser
100    105    110
Ala Leu Val Lys Ala Cys Asn Ala Tyr Phe Lys Leu Gly Leu Asn Arg
115    120    125
Ser Gln Leu Ala Gln Glu Ala Lys Phe Ala Ser Gly Ser Ser Ser Arg
130    135    140
Ser Phe Tyr Gly Pro Leu Gly Ala Trp Asp Lys Asp Ser Gly Glu Ile
145    150    155    160
Tyr Pro Val Glu Thr Gly Leu Lys Leu Ala Met Ile Met Leu Val Leu
165    170    175
Glu Asp Lys Lys Lys Pro Ile Ser Ser Arg Asp Gly Met Lys Leu Cys
180    185    190
Val Glu Thr Ser Thr Thr Phe Asp Asp Trp Val Arg Gln Ser Glu Lys
195    200    205
Asp Tyr Gln Asp Met Leu Val Tyr Leu Lys Ala Asn Asp Phe Ala Lys
210    215    220
Val Gly Glu Leu Thr Glu Lys Asn Ala Leu Ala Met His Ala Thr Thr
225    230    235    240

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Lys Thr Ala Ser Pro Ala Phe Ser Tyr Leu Thr Asp Ala Ser Tyr Glu
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Ala Met Asp Phe Val Arg Gln Leu Arg Glu Gln Gly Glu Ala Cys Tyr
                260                265                270
Phe Thr Met Asp Ala Gly Pro Asn Val Lys Val Leu Cys Gln Glu Lys
                275                280                285
Asp Leu Glu His Leu Ser Glu Ile Phe Gly Gln Arg Tyr Arg Leu Ile
                290                295                300
Val Ser Lys Thr Lys Asp Leu Ser Gln Asp Gly Cys Cys
305                310                315

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<210> 2

<211> 317

<212> PRT

<213> Artificial Sequence

<220>

<223> Mutant 2B4 of MDP decarboxylase

<400> 2

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20          25          30
Ser Ser Ile Ser Leu Thr Leu Glu Asn Met Tyr Thr Glu Thr Thr Leu
35          40          45
Ser Ser Leu Pro Thr Asp Ala Thr Ala Asp Ala Phe Tyr Ile Asn Gly
50          55          60
Gln Leu Gln Asn Glu Ala Glu His Val Lys Met Ser Lys Ile Ile Asp
65          70          75          80
Arg Tyr Arg Pro Asp Gly Asp Gly Phe Val Arg Ile Asp Thr Gln Asn
85          90          95
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100         105         110
Ala Leu Val Lys Ala Cys Asn Ala Arg Phe Lys Leu Gly Leu Asn Arg
115         120         125
Ser Gln Leu Ala Gln Glu Ala Lys Phe Ala Ser Gly Ser Ser Ser Arg
130         135         140
Ser Phe Tyr Gly Pro Leu Gly Ala Trp Asp Lys Asp Ser Gly Leu Ile
145         150         155         160
Tyr Pro Val Glu Thr Gly Leu Lys Leu Ala Met Ile Cys Leu Val Leu
165         170         175
Glu Asp Lys Lys Lys Pro Ile Ser Ser Arg Asp Gly Met Lys Leu Cys
180         185         190
Val Glu Thr Ser Thr Thr Phe Asp Asp Trp Val Arg Gln Ser Glu Lys
195         200         205
Asp Tyr Gln Asp Met Leu Val Tyr Leu Lys Ala Asn Asp Phe Ala Lys
210         215         220
Val Gly Glu Leu Thr Glu Lys Asn Ala Leu Ala Met His Ala Thr Thr
225         230         235         240
Lys Thr Ala Ser Pro Ala Phe Ser Tyr Leu Thr Asp Ala Ser Tyr Glu
245         250         255
Ala Met Asp Phe Val Arg Gln Leu Arg Glu Gln Gly Glu Ala Cys Tyr
260         265         270
Phe Thr Met Asp Ala Gly Pro Asn Val Cys Val Leu Cys Gln Glu Lys
275         280         285
Asp Leu Glu His Leu Ser Glu Ile Phe Gly Gln Arg Tyr Arg Met Ile
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305         310         315

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<210> 3

<211> 317

<212> PRT

<213> Artificial Sequence

<220>

<223> Mutant F9 of MDP decarboxylase

<400> 3

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                20                25                30
Ser Ser Ile Ser Leu Thr Leu Glu Asn Met Tyr Thr Glu Thr Thr Leu
35          40          45

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Ser Ser Leu Pro Thr Asp Ala Thr Ala Asp Ala Phe Tyr Ile Asn Gly
50      55      60
Gln Leu Gln Asn Glu Ala Glu His Val Lys Met Ser Lys Ile Ile Asp
65      70      75      80
Arg Tyr Arg Pro Asp Gly Asp Gly Phe Val Arg Ile Asp Thr Gln Asn
85      90      95
Ser Met Pro Thr Ala Ala Gly Leu Ser Ser Ser Ser Gly Leu Ser
100     105     110
Ala Leu Val Lys Ala Leu Asn Ala Arg Phe Lys Leu Gly Leu Asn Arg
115     120     125
Ser Gln Leu Ala Gln Glu Ala Lys Phe Ala Ser Gly Ser Ser Ser Arg
130     135     140
Ser Phe Tyr Gly Pro Leu Gly Ala Trp Asp Lys Asp Ser Gly Leu Ile
145     150     155     160
Tyr Pro Val Glu Thr Gly Leu Lys Leu Ala Met Ile Cys Leu Val Leu
165     170     175
Cys Asp Lys Lys Lys Pro Ile Ser Ser Arg Asp Gly Met Lys Leu Cys
180     185     190
Val Glu Thr Ser Thr Thr Phe Asp Asp Trp Val Arg Gln Ser Glu Lys
195     200     205
Asp Tyr Gln Asp Met Leu Val Tyr Leu Lys Ala Asn Asp Phe Ala Lys
210     215     220
Val Gly Glu Leu Thr Glu Lys Asn Ala Leu Ala Met His Ala Thr Thr
225     230     235     240
Lys Thr Ala Ser Pro Ala Phe Ser Tyr Leu Thr Asp Ala Ser Tyr Glu
245     250     255
Ala Met Asp Phe Val Arg Gln Leu Arg Glu Gln Gly Glu Ala Cys Tyr
260     265     270
Phe Thr Met Asp Ala Gly Pro Asn Val Cys Val Leu Cys Gln Glu Lys
275     280     285
Asp Leu Asp His Leu Ser Glu Ile Leu Gly Gln Arg Tyr Arg Met Ile
290     295     300
Val Ser Lys Ser Lys Asp Leu Ser Gln Asp Gly Cys Cys
305     310     315

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<211> 957

<212> DNA

<213> Streptococcus mitis

<220>

<221> source

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<223> /mol_type="unassigned DNA" /organism="Streptococcus mitis"

<400> 4

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aatatgtata ccgaaaccac cctgagcagc ctgccgaccg atgcaaccgc agatgcattt      180
tatattaatg gtcagctgca gaacgaagcc gaacatgtta aaatgagcaa aatcatcgat      240
cgctatcgtc cggatggtga tggttttggt cgtattgata ccagaatag tatgccgacc      300
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gattttgcca aagttggtga gctgaccgaa aaaaatgcac tggcaatgca cgcaaccacc      720
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PATENTKRAV

1. Variant af en mevalonat-diphosphat-decarboxylase, som har en forbedret aktivitet ved omsætning af 3-phosphonoxyisovalerat til isobuten i forhold til mevalonat-diphosphat-decarboxylasen ifølge SEQ ID NO:1, hvor mevalonat-diphosphat-decarboxylase-varianten er **kendetegnet ved, at** den har en aminosyresekvens, som angivet i SEQ ID NO:1 eller en aminosyresekvens med i det mindste 60% sekvensidentitet med SEQ ID NO:1, hvori aminosyreresten ved position 282 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til den nævnte position i den nævnte sekvens, som har i det mindste 60% sekvensidentitet med SEQ ID NO:1, er substitueret med en anden aminosyrerest.

2. Mevalonat-diphosphat-decarboxylase-variant ifølge krav 1, hvor: aminosyreresten ved position 282 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i den nævnte sekvens, som har i det mindste 60% sekvensidentitet med SEQ ID NO:1, er deleteret eller substitueret med cystein, serin, glutaminsyre, glycin, glutamin, threonin, valin, alanin eller asparaginsyre.

3. Mevalonat-diphosphat-decarboxylase-variant ifølge krav 1 eller 2, hvor i det mindste én yderligere aminosyrerest er substitueret eller deleteret ved en position valgt fra gruppen bestående af positionerne 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267 og 315 i SEQ ID NO:1 eller ved en position svarende til denne position i den nævnte sekvens, som har i det mindste 60% sekvensidentitet med SEQ ID NO:1.

4. Mevalonat-diphosphat-decarboxylase-variant ifølge krav 3, hvor aminosyreresten ved position 282 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i den nævnte sekvens, som har i det mindste 60% sekvensidentitet med SEQ ID NO:1, er deleteret eller substitueret med en anden aminosyrerest og hvor aminosyreresten ved position 121 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i den nævnte sekvens, som har i det mindste 60% sekvensidentitet med SEQ ID NO:1 er deleteret eller substitueret

med en anden aminosyrerest.

5. Mevalonat-diphosphat-decarboxylase-variant ifølge krav 3, hvor:

- (1) en aminosyrerest ved position 1 i aminosyresekvensen vist i SEQ ID NO:1 eller
5 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med leucin; og/eller
- (2) en aminosyrerest ved position 2 i aminosyresekvensen vist i SEQ ID NO:1 eller
ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med histidin; og/eller
- 10 (3) en aminosyrerest ved position 9 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med leucin; og/eller
- (4) en aminosyrerest ved position 11 i aminosyresekvensen vist i SEQ ID NO:1 eller
ved en position svarende til denne position i en relateret sekvens, er deleteret eller
15 substitueret med cystein, glutaminsyre eller phenylalanin, fortrinsvis cystein; og/eller
- (5) en aminosyrerest ved position 16 i aminosyresekvensen vist i SEQ ID NO:1 eller
ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med leucin; og/eller
- (6) en aminosyrerest ved position 23 i aminosyresekvensen vist i SEQ ID NO:1 eller
20 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med leucin; og/eller
- (7) en aminosyrerest ved position 24 i aminosyresekvensen vist i SEQ ID NO:1 eller
ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med arginin, serin eller leucin; og/eller
- 25 (8) en aminosyrerest ved position 28 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med lysin eller alanin; og/eller
- (9) en aminosyrerest ved position 31 i aminosyresekvensen vist i SEQ ID NO:1 eller
ved en position svarende til denne position i en relateret sekvens, er deleteret eller
30 substitueret med serin; og/eller
- (10) en aminosyrerest ved position 42 i aminosyresekvensen vist i SEQ ID NO:1 eller
ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med alanin eller leucin; og/eller
- (11) en aminosyrerest ved position 43 i aminosyresekvensen vist i SEQ ID NO:1 eller
35 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med leucin; og/eller

- (12) en aminosyrerest ved position 45 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med leucin, phenylalanin, methionin eller valin, fortrinsvis leucin; og/eller
- 5 (13) en aminosyrerest ved position 53 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med valin; og/eller
- (14) en aminosyrerest ved position 57 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med serin; og/eller
- 10 (15) en aminosyrerest ved position 58 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med threonin; og/eller
- (16) en aminosyrerest ved position 66 i aminosyresekvensen vist i SEQ ID NO:1 ved en position svarende til denne position i en relateret sekvens, er deleteret eller
- 15 substitueret med histidin; og/eller
- (17) en aminosyrerest ved position 75 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med isoleucin; og/eller
- (18) en aminosyrerest ved position 77 i aminosyresekvensen vist i SEQ ID NO:1 eller
- 20 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med asparagin eller arginin; og/eller
- (19) en aminosyrerest ved position 80 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med glycin; og/eller
- 25 (20) en aminosyrerest ved position 86 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med glutamin; og/eller
- (21) en aminosyrerest ved position 87 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller
- 30 substitueret med glutaminsyre; og/eller
- (22) en aminosyrerest ved position 91 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med histidin; og/eller
- (23) en aminosyrerest ved position 105 i aminosyresekvensen vist i SEQ ID NO:1 eller
- 35 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med alanin; og/eller

- (24) en aminosyrerest ved position 111 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med methionin; og/eller
- 5 (25) en aminosyrerest ved position 116 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med arginin, isoleucin, leucin, serin eller methionin, fortrinsvis arginin eller isoleucin; og/eller
- (26) en aminosyrerest ved position 118 i aminosyresekvensen vist i SEQ ID NO:1 ved en position svarende til denne position i en relateret sekvens, er deleteret eller
- 10 substitueret med leucin eller tryptophan; og/eller
- (27) en aminosyrerest ved position 120 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med asparagin, leucin, arginin, isoleucin eller valin, fortrinsvis asparagin, leucin, arginin eller isoleucin; og/eller
- 15 (28) en aminosyrerest ved position 121 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med arginin, leucin, tryptophan, phenylalanin, tyrosin, asparagin eller lysin, fortrinsvis arginin eller phenylalanin; og/eller
- (29) aminosyreresten at position 122 i aminosyresekvensen vist i SEQ ID NO:1 eller
- 20 ved en position svarende til denne position i en relateret sekvens, er substitueret med methionin eller tyrosin; og/eller
- (30) en aminosyrerest ved position 123 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med methionin eller arginin; og/eller
- 25 (31) en aminosyrerest ved position 129 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med prolin eller valin; og/eller
- (32) en aminosyrerest ved position 134 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller
- 30 substitueret med glycin; og/eller
- (33) en aminosyrerest ved position 139 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med cystein eller alanin; og/eller
- (34) en aminosyrerest ved position 141 i aminosyresekvensen vist i SEQ ID NO:1 eller
- 35 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med prolin, cystein, glycin eller threonin; og/eller

- (35) en aminosyrerest ved position 142 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med alanin; og/eller
- 5 (36) en aminosyrerest ved position 159 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med leucin; og/eller
- (37) en aminosyrerest ved position 160 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med valin; og/eller
- 10 (38) en aminosyrerest ved position 161 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med arginin; og/eller
- (39) en aminosyrerest ved position 164 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller
- 15 substitueret med glutamin; og/eller
- (40) en aminosyrerest ved position 166 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med serin; og/eller
- (41) en aminosyrerest ved position 173 i aminosyresekvensen vist i SEQ ID NO:1 eller
- 20 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med cystein; og/eller
- (42) en aminosyrerest ved position 177 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med valin eller cystein, fortrinsvis valin; og/eller
- 25 (43) en aminosyrerest ved position 179 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med lysin eller leucin; og/eller
- (44) en aminosyrerest ved position 180 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller
- 30 substitueret med prolin; og/eller
- (45) en aminosyrerest ved position 182 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med glutaminsyre; og/eller
- (46) en aminosyrerest ved position 186 i aminosyresekvensen vist i SEQ ID NO:1 eller
- 35 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med histidin, leucin, valin, isoleucin eller asparagin; og/eller

- (47) en aminosyrerest ved position 188 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med cystein; og/eller
- (48) en aminosyrerest ved position 198 i aminosyresekvensen vist i SEQ ID NO:1 eller
5 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med asparaginsyre; og/eller
- (49) en aminosyrerest ved position 204 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med leucin; og/eller
- 10 (50) en aminosyrerest ved position 205 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med histidin; og/eller
- (51) en aminosyrerest ved position 208 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller
15 substitueret med leucin; og/eller
- (52) en aminosyrerest ved position 215 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med alanin; og/eller
- (53) en aminosyrerest ved position 221 i aminosyresekvensen vist i SEQ ID NO:1 eller
20 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med glutaminsyre; og/eller
- (54) en aminosyrerest ved position 227 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med lysin; og/eller
- 25 (55) en aminosyrerest ved position 231 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med glutamin eller leucin; og/eller
- (56) en aminosyrerest ved position 238 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller
30 substitueret med arginin, glutaminsyre eller lysin; og/eller
- (57) en aminosyrerest ved position 241 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med methionin eller isoleucin; og/eller
- (58) en aminosyrerest ved position 242 i aminosyresekvensen vist i SEQ ID NO:1 eller
35 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med alanin eller glutaminsyre; og/eller

- (59) en aminosyrerest ved position 246 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med glutaminsyre; og/eller
- 5 (60) en aminosyrerest ved position 248 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med threonin; og/eller
- (61) en aminosyrerest ved position 251 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med methionin, phenylalanin eller valin, fortrinsvis methionin; og/eller
- 10 (62) en aminosyrerest ved position 252 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med glutaminsyre; og/eller
- (63) en aminosyrerest ved position 253 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller
- 15 substitueret med valin eller isoleucin; og/eller
- (64) en aminosyrerest ved position 255 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med glutaminsyre; og/eller
- (65) en aminosyrerest ved position 258 i aminosyresekvensen vist i SEQ ID NO:1 eller
- 20 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med leucin; og/eller
- (66) en aminosyrerest ved position 264 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med glutamin; og/eller
- 25 (67) en aminosyrerest ved position 267 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med arginin; og/eller
- (68) en aminosyrerest ved position 279 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller
- 30 substitueret med alanin; og/eller
- (69) en aminosyrerest ved position 282 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med cystein, serin, glutaminsyre, glycin, glutamin, threonin, valin, alanin eller asparaginsyre; og/eller

(70) en aminosyrerest ved position 291 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med asparaginsyre; og/eller

5 (71) en aminosyrerest ved position 293 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med phenylalanin eller tryptophan; og/eller

(72) en aminosyrerest ved position 297 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med cystein eller leucin, fortrinsvis cystein; og/eller

10 (73) en aminosyrerest ved position 299 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med prolin eller lysin; og/eller

(74) en aminosyrerest ved position 303 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller
15 substitueret med methionin; og/eller

(75) en aminosyrerest ved position 307 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med histidin; og/eller

(76) en aminosyrerest ved position 308 i aminosyresekvensen vist i SEQ ID NO:1 eller
20 ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med serin; og/eller

(77) en aminosyrerest ved position 315 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til denne position i en relateret sekvens, er deleteret eller substitueret med serin.

25

6. Mevalonat-diphosphat-decarboxylase-variant ifølge ethvert af kravene 1 til 5, som har en aminosyresekvens som vist i SEQ ID NO: 1, hvor følgende substitutioner er effektuerede:

30 (i) K24R-C118L-Y121R-E159L-M173C-E177C-K282C-E291D-F297L-L303M-T308S; eller
(ii) Y121R-E159L-M173C-K282C-L303M-T308S.

7. Mevalonat-diphosphat-decarboxylase-variant ifølge krav 6 (i), som har følgende
35 yderligere substitutioner:

S141P-I16L-K241I-S248T-M28K-K180P; eller
S141P-I16L-R91H-K241M-S248T-Q299K.

8. Nucleinsyremolekyle, som koder for mevalonat-diphosphat-decarboxylase-varianten
5 ifølge ethvert af kravene 1 til 7.

9. Vektor omfattende nucleinsyremolekylet ifølge krav 8.

10. Værtscelle omfattende vektoren ifølge krav 9.

10

11. Fremgangsmåde til tilvejebringelse af en variant af en mevalonat-diphosphat-decarboxylase, hvor varianten har en forbedret aktivitet til konvertering af 3-phosphonoxysovalerat til isobuten i forhold til mevalonat-diphosphat-decarboxylasen ifølge SEQ ID NO:1, hvilken fremgangsmåde omfatter trinnet med at effektuere en ændring
15 ved aminosyrepositionen 282 i aminosyresekvensen vist i SEQ ID NO:1 eller ved en position svarende til den nævnte position i en aminosyresekvens, som har i det mindste 60% sekvensidentitet med SEQ ID NO: 1, hvor ændringen er en substitution eller deletion.

20 12. Fremgangsmåde ifølge krav 11, hvor fremgangsmåden yderligere omfatter trinnet med at effektuere én eller flere yderligere ændringer i sekvensen for mevalonat-diphosphat-decarboxylasen, hvor ændringen/ændringerne effektueres ved én eller flere aminosyrepositioner valgt fra gruppen bestående af aminosyrepositionerne 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91, 105, 116, 118, 120, 121, 122, 123, 129, 134,
25 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308, 1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267 og 315 i aminosyresekvensen vist i SEQ ID NO:1, eller ved en position svarende til en vilkårlig enkelt af disse positioner i den nævnte sekvens, som har i det mindste 60% sek-
30 vensidentitet med SEQ ID NO:1.

13. Fremgangsmåde ifølge krav 11 eller 12, hvor ændringerne, som effektueres i en mevalonat-diphosphat-decarboxylase ved én eller flere positioner svarende til positionerne svarende til positionerne 282, 9, 11, 16, 24, 28, 42, 43, 45, 53, 66, 77, 80, 91,
35 105, 116, 118, 120, 121, 122, 123, 129, 134, 141, 159, 160, 161, 173, 177, 180, 186, 215, 238, 241, 242, 248, 251, 253, 258, 264, 279, 291, 293, 297, 299, 303, 307, 308,

1, 2, 23, 31, 57, 58, 75, 86, 87, 111, 139, 142, 164, 166, 179, 182, 188, 198, 204, 205, 208, 221, 227, 231, 246, 252, 255, 267 og 315 i aminosyresekvensen vist i SEQ ID NO:1 er valgt fra gruppen bestående af disse, som er identificerede i nr. (1) til (77) i krav 5.

5

14. Anvendelse af mevalonat-diphosphat-decarboxylasen ifølge ethvert af kravene 1 til 7 eller værtscellen ifølge krav 10 til

- 10
- (i) konvertering af 3-hydroxyisovalerat eller af 3-phosphonoxisovalerat til isobuten;
 - (ii) konverteringen af mevalonat eller af mevalonat-3-phosphat til isoprenol; eller
 - (iii) konverteringen af 3-hydroxypent-4-enoat eller af 3-phosphonoxypent-4-enoat til 1,3-butadien.

15 **15.** Fremgangsmåde til fremstilling af isobuten ud fra 3-hydroxyisovalerat eller ud fra 3-phosphonoxisovalerat, omfattende trinnene med:

- (i) dyrkning af værtscellen ifølge krav 10 i et passende medie; og
- (ii) udvinding af den fremstillede isobuten.

20

16. Fremgangsmåde til fremstilling af isoprenol ud fra mevalonat eller ud fra mevalonat-3-phosphat, omfattende trinnene med:

- 25
- (i) dyrkning af værtscellen ifølge krav 10 i et passende medie; og
 - (ii) udvinding af den fremstillede isoprenol.

17. Fremgangsmåde til fremstilling af 1,3-butadien ud fra 3-hydroxypent-4-enoat eller ud fra 3-phosphonoxypent-4-enoat, omfattende trinnene med:

- 30
- (i) dyrkning af værtscellen ifølge krav 10 i et passende medie; og
 - (ii) udvinding af den fremstillede 1,3-butadien.

DRAWINGS

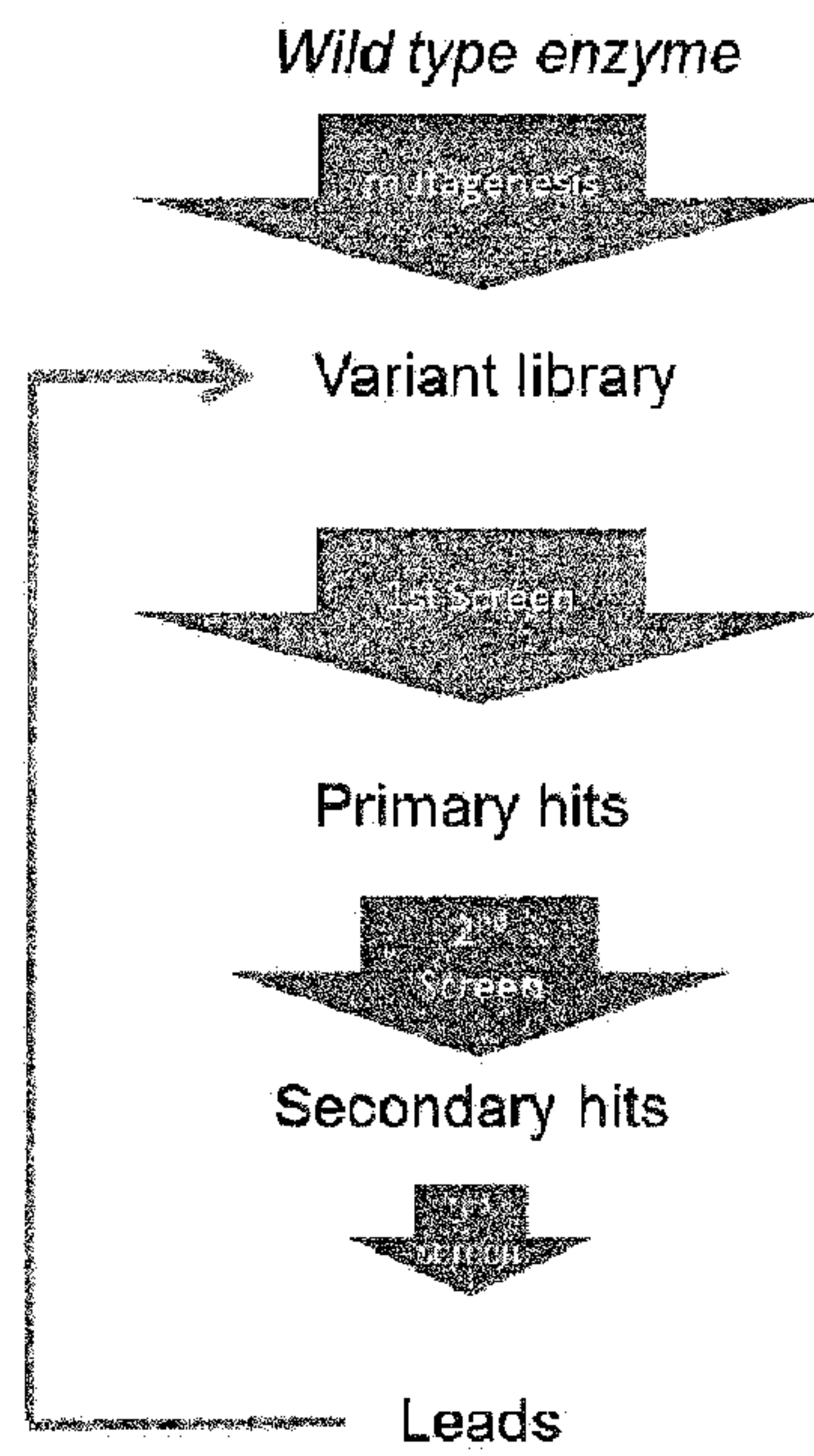


Figure 1

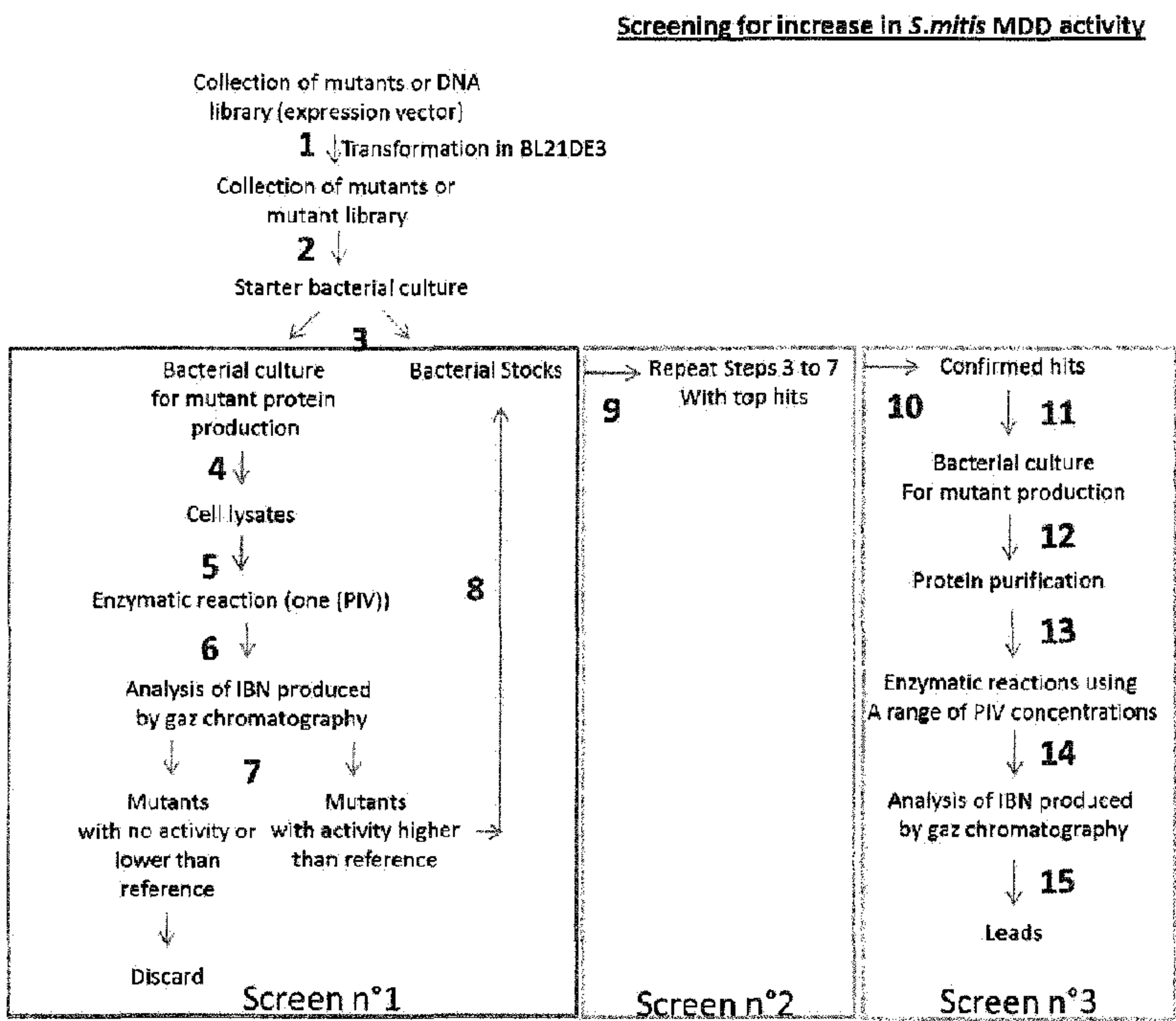


Figure 2

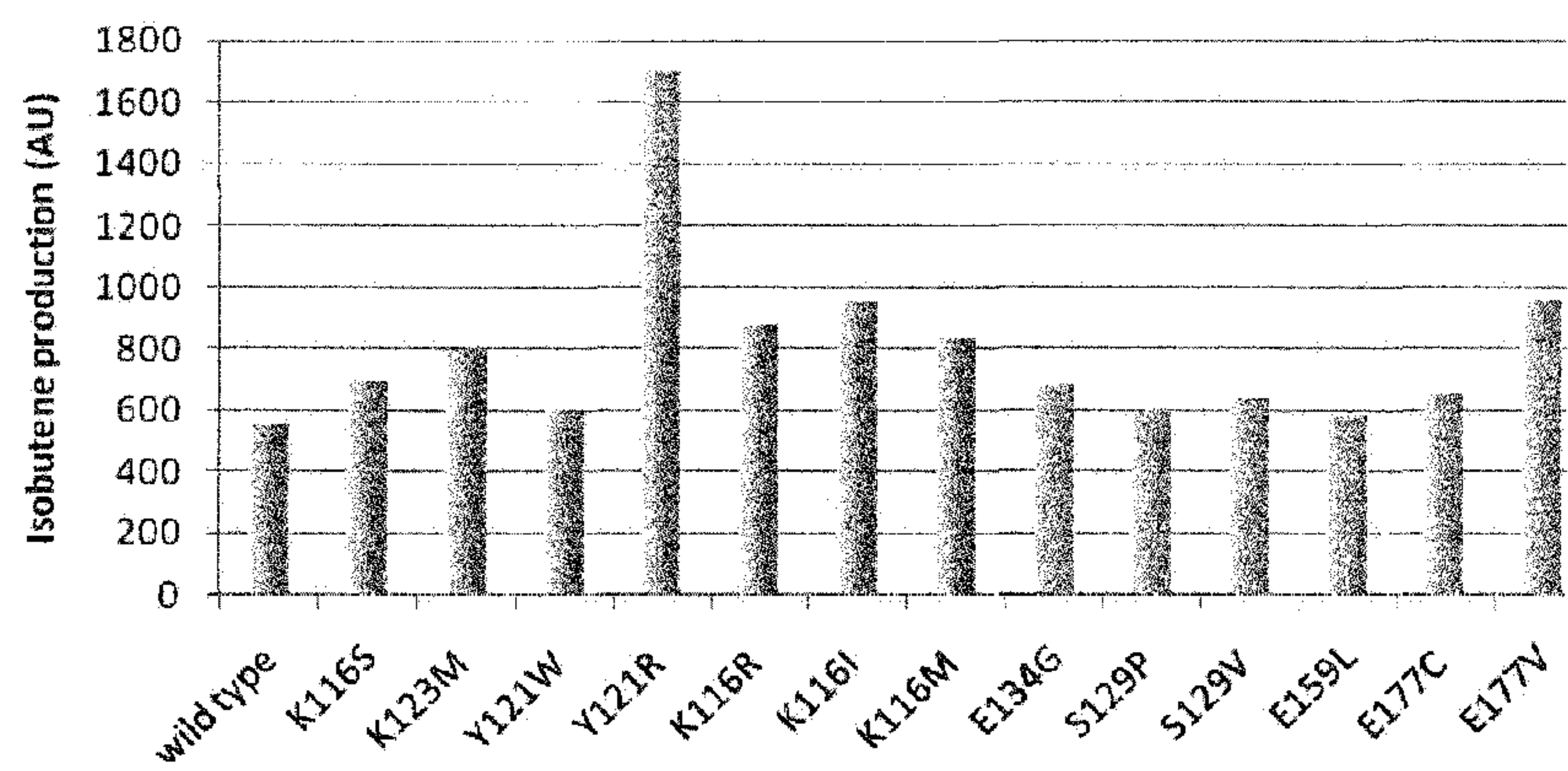


Figure 3

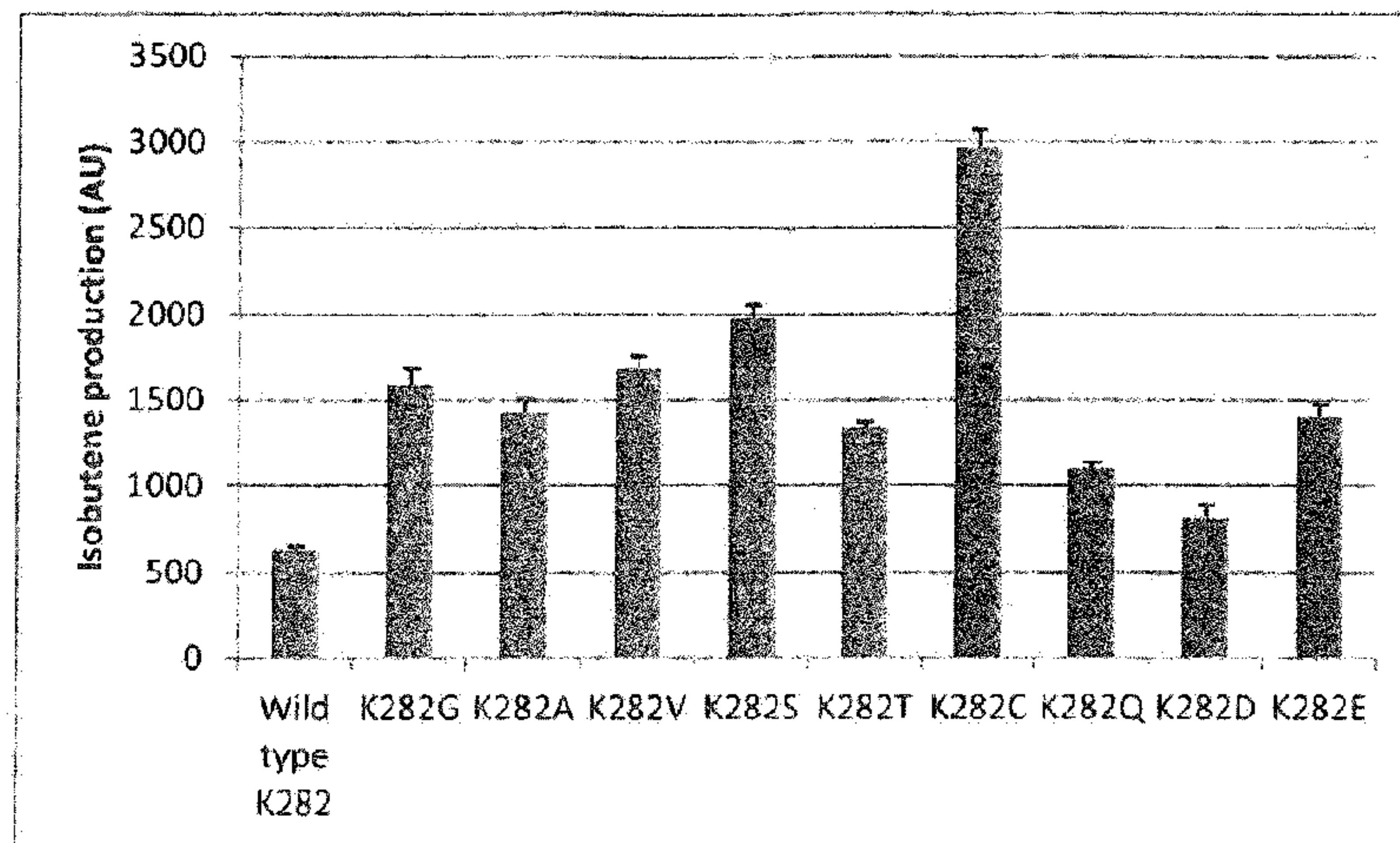


Figure 4a

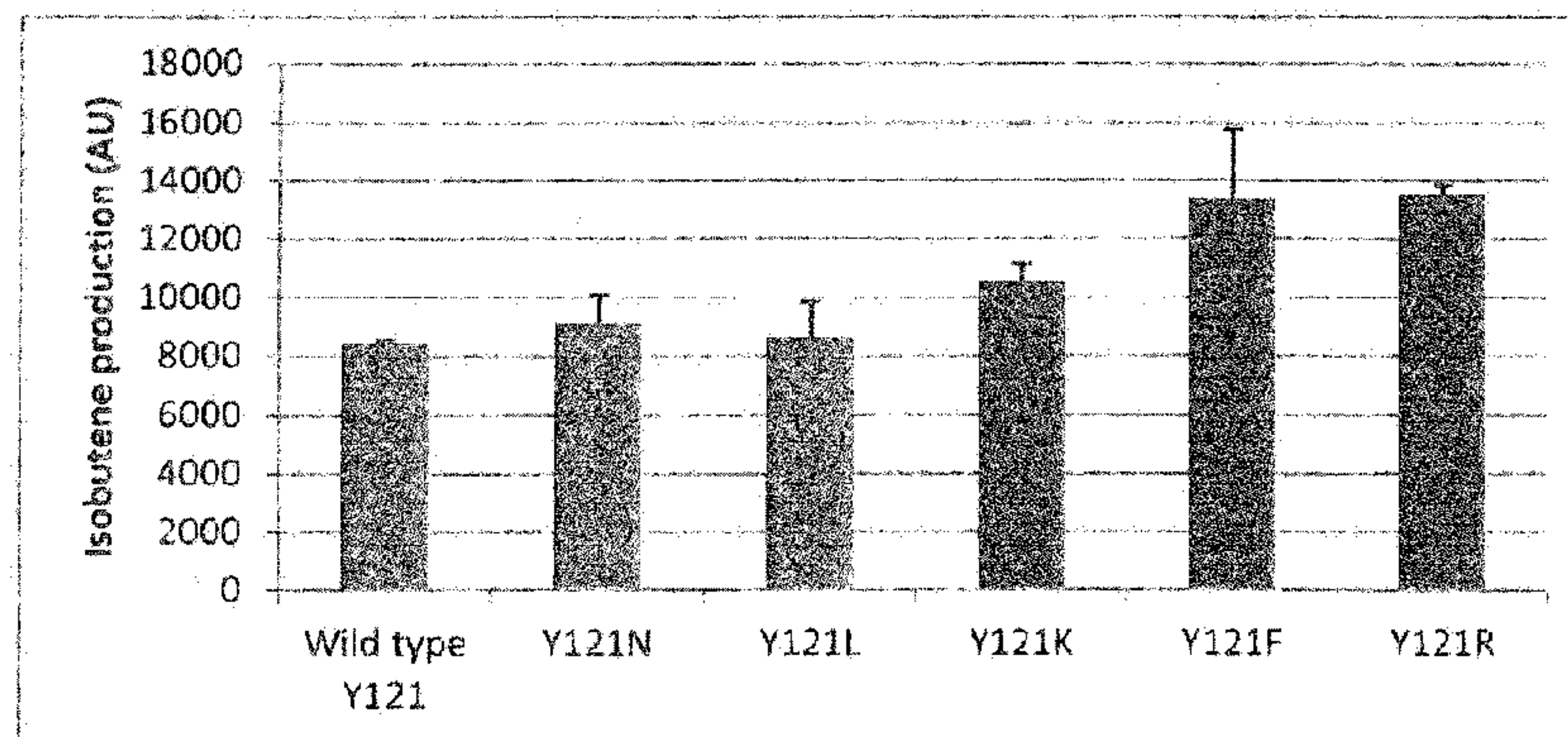


Figure 4b

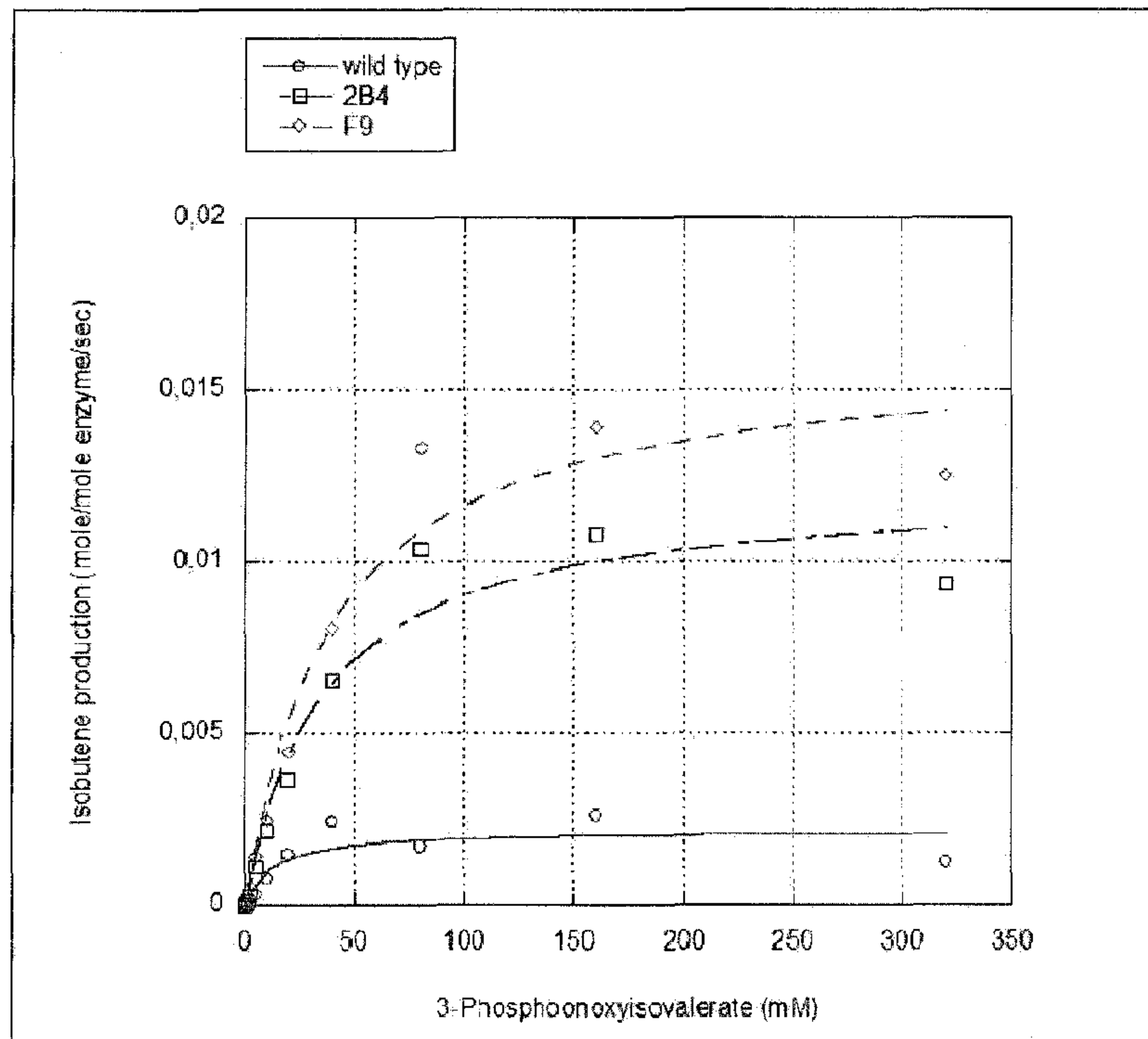


Figure 5

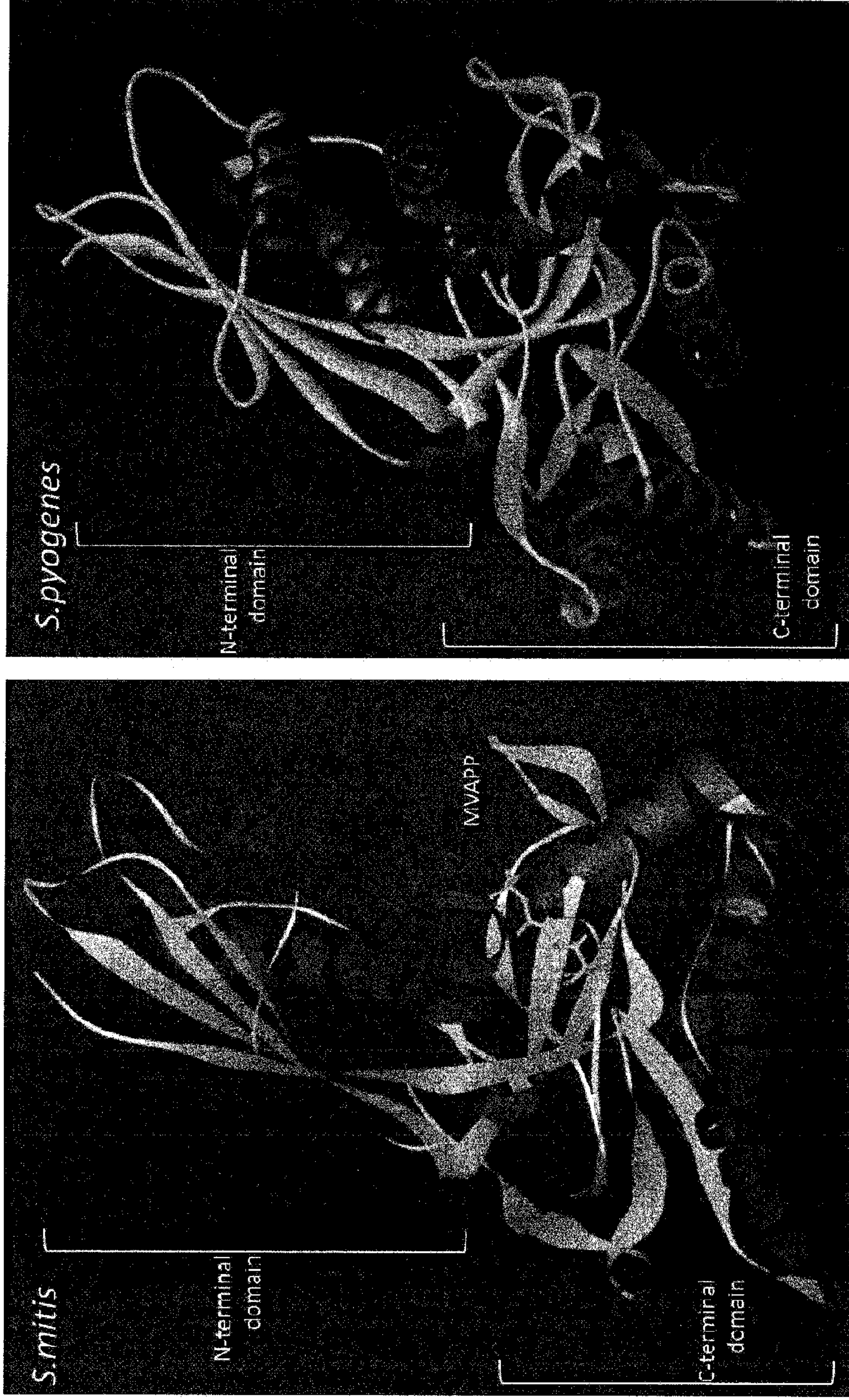


Figure 6

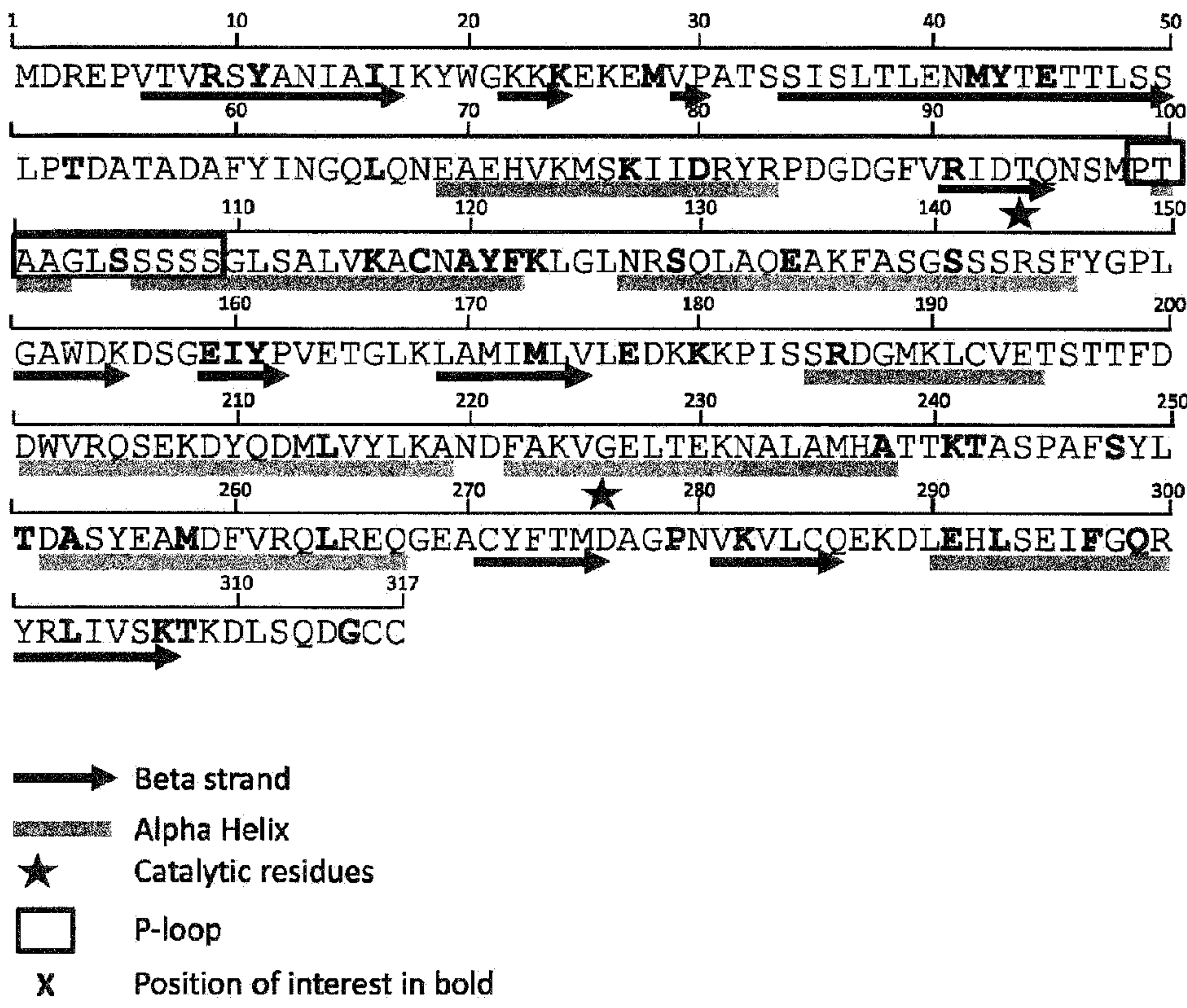
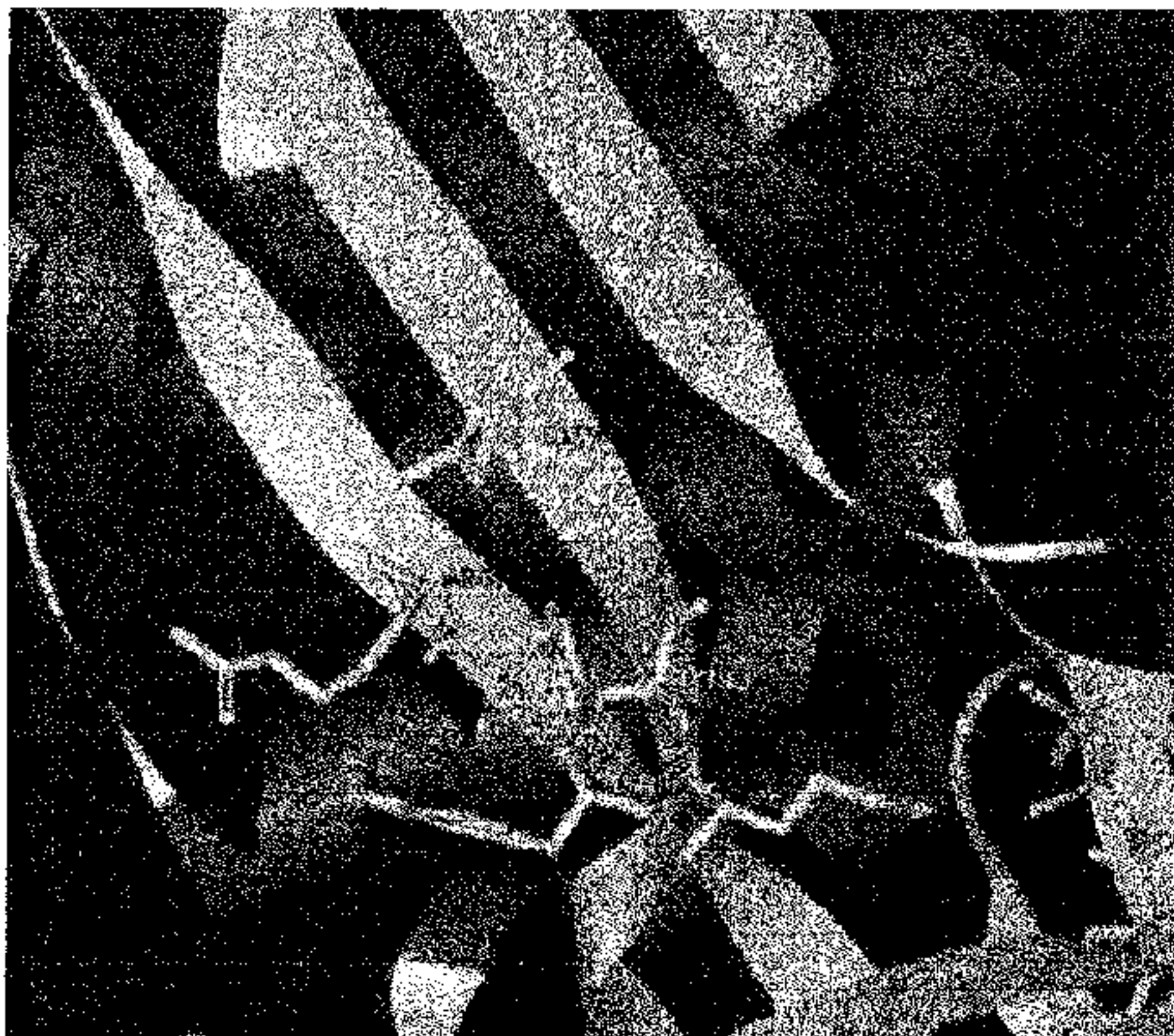


Figure 7

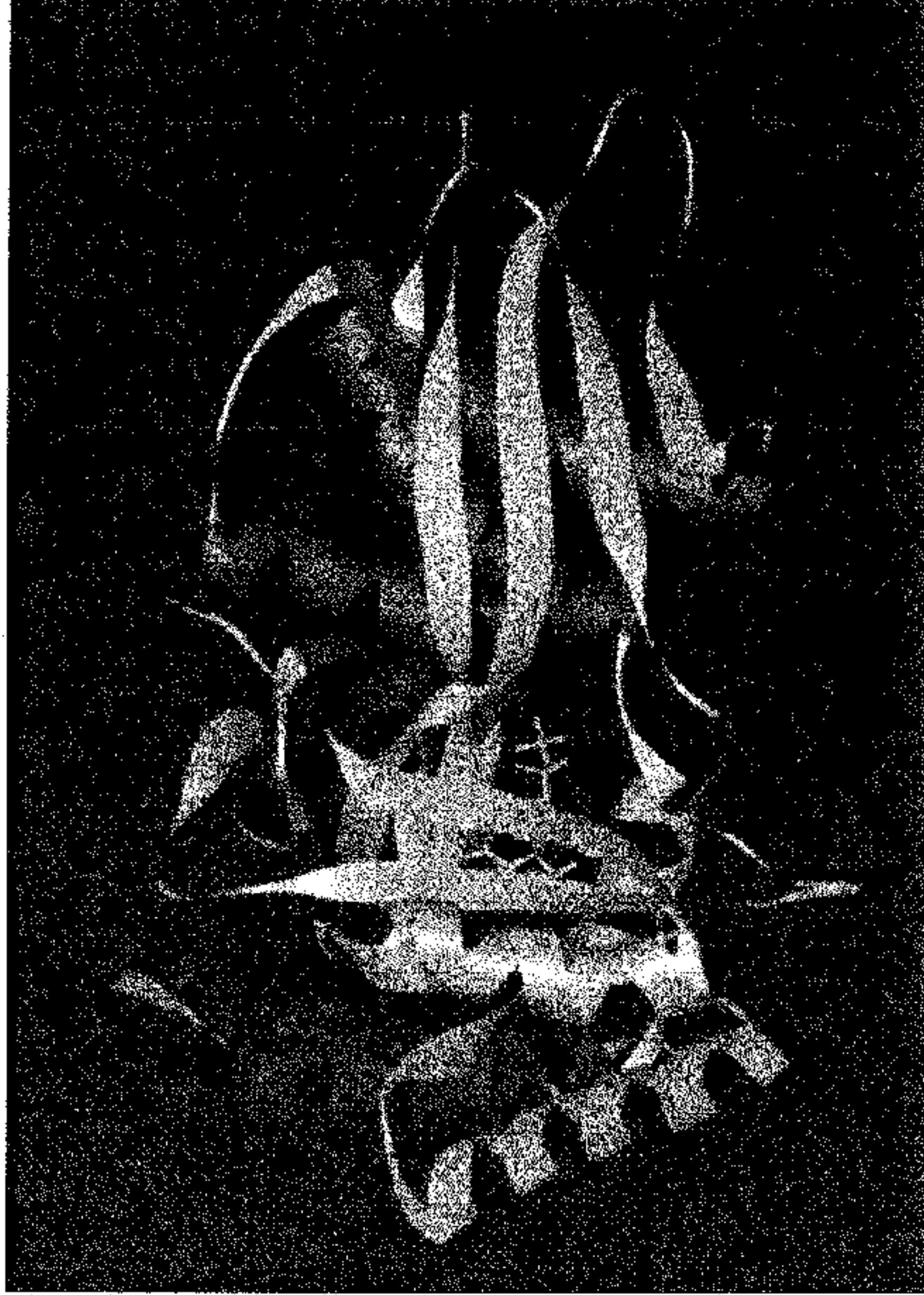


A



B

Figure 8



C

Figure 8 (continued)

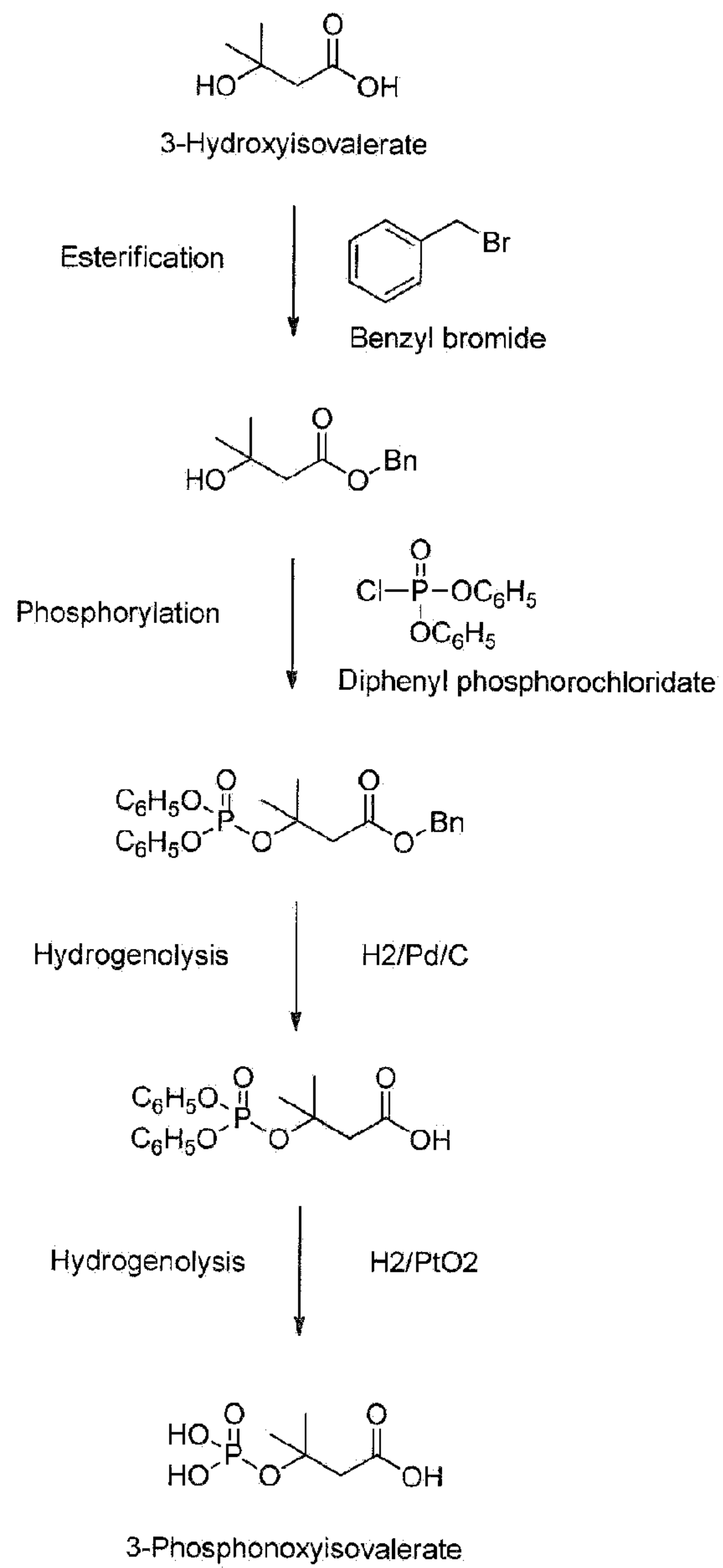


Figure 9

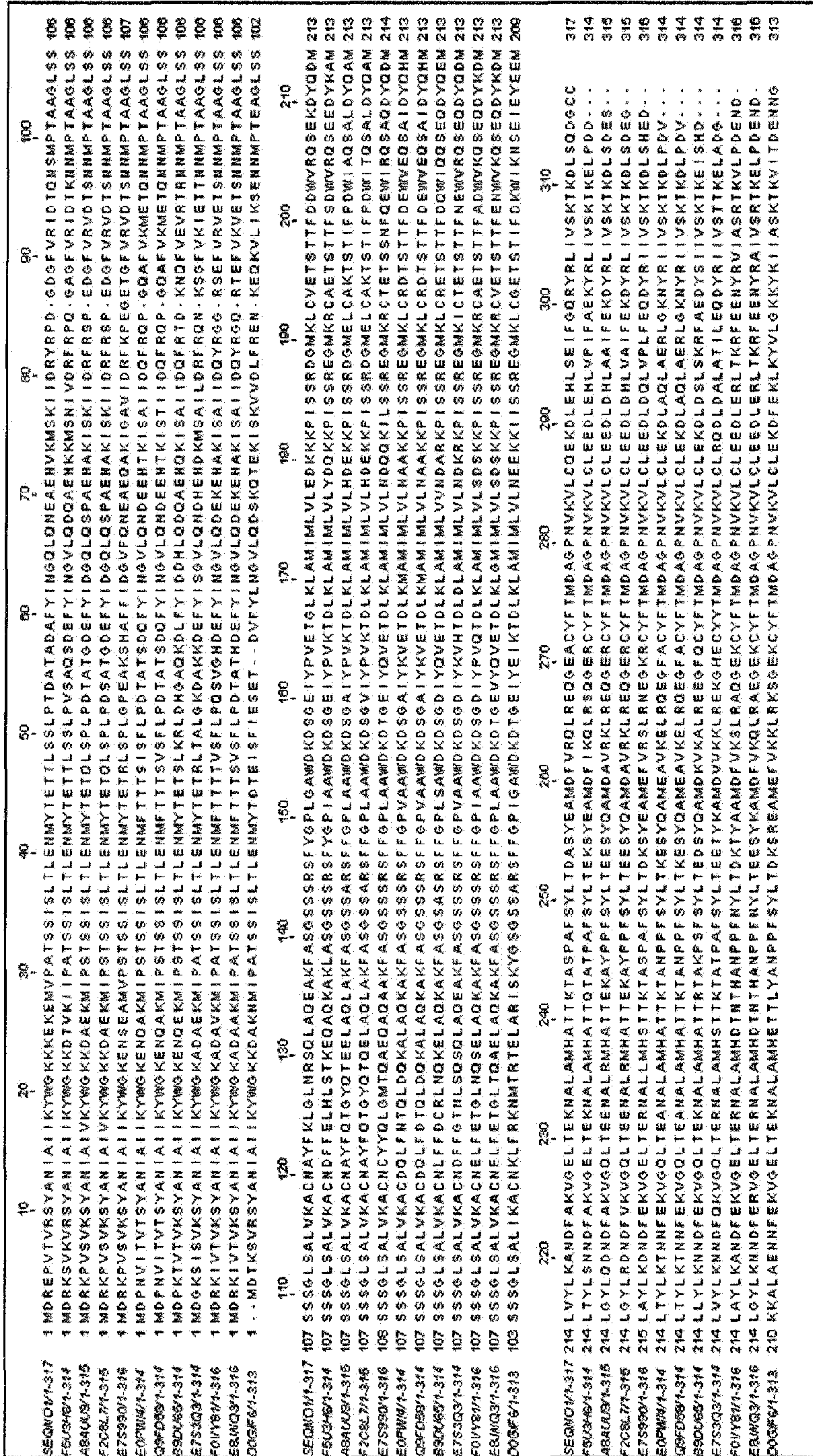


Figure 10

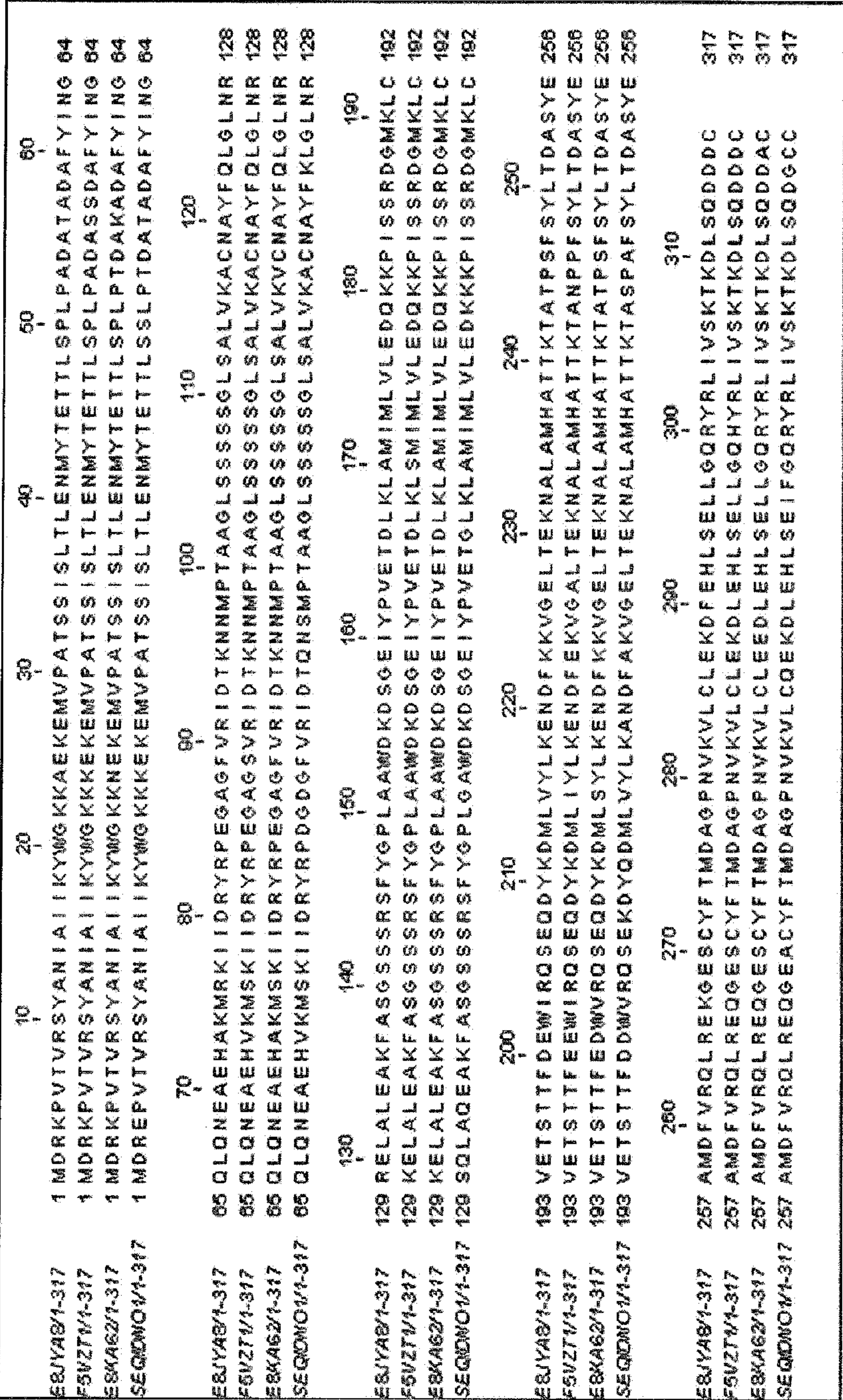


Figure 11

SEQNO 71-317	10	20	30	40	50	60	70	
E1LG71-317	1	MDREPVTVRSYANIAI	IKYWGKKKKEKEMVPATSSISLTLENMYTETITLSSLP	TADATADAFYINGQLQNEAEHVKMSKI	ID	80		
E6KKU6-317	1	MDREPVTVRSYANIAI	IKYWGKKKKEKEMVPATSSISLTLENMYTETITLSSLP	TADATADAFYINGQLQNEAEHVKMSKI	ID	80		
D4FR73-317	1	MDRKPVTVRSYANIAI	IKYWGKKKKEKEMVPATSSISLTLENMYTETITLSSLP	TADATADAFYINGQLQNEAEHVKMSKI	ID	80		
E9FW52-28-344	28	MDREPVTVRSYANIAI	IKYWGKKKKEKEMVPATSSISLTLENMYTETITLSSLP	TADATADAFYINGQLQNEAEHVKMSKI	ID	107		
Q8DR50-28-344	28	MDREPVTVRSYANIAI	IKYWGKKKKEKEMVPATSSISLTLENMYTETITLSSLP	PANVTADAFYINGQLQNEAEHVKMSKI	ID	107		
SEQNO 71-317	80	100	110	120	130	140	150	
E1LG71-317	81	RYRPDGGFVRIDTQNSMPTAAOLSSSSSGLSALVKACNAYFKLGLNRSQ	LAQEAKFASGSSSRSFYGPLGAWDKDSGEI	160				
E6KKU6-317	81	RYRPAGEGFIRIDTONNMPTAAOLSSSSSGLSALVKACNAYFKLGLDRSQ	LAQEAKFASGSSSRSFYGPLGAWDKDSGEI	160				
D4FR73-317	81	RYRPAGEGFVRIDTQNNMPTAAOLSSSSSGLSALVKACNAYFKLGLNRSQ	LAQEAKFASGSSSRSFYGPLGAWDKDSGEI	160				
E9FW52-28-344	81	RYRPEGEGFVRIDTQNNMPTAAOLSSSSSGLSALVKACNAYFKLGLDRSQ	LAQEAKFASGSSSRSFYGPLGAWDKDSGEI	160				
Q8DR50-28-344	108	RYRPAGEGFVRIDTQNNMPTAAOLSSSSSGLSALVKACNAYFKLGLDRSQ	LAQEAKFASGSSSRSFYGPLGAWDKDSGEI	187				
SEQNO 71-317	170	180	190	200	210	220	230	
E1LG71-317	181	YPVETDLKLAMIMLVLEDK	KKKPISSRDGMKLCVETSTTFFDDMWVRQSEKDYQDMLVYLKANDFAKVGE	LTEKNALAMHATT	240			
E6KKU6-317	181	YPVETDLKLAMIMLVLEDK	KKKPISSRDGMKLCVETSTTFFDDMWVRQSEKDYQDMLVYLKENDFTKVGE	LTEKNALAMHATT	240			
D4FR73-317	181	YPVETDLRLAMIMLVLEDK	KKKPISSRDGMKLCVETSTTFFDDMWVRQSEKDYQDMLVYLKENDFANVGE	LTEKNALAMHATT	240			
E9FW52-28-344	181	YPVETDLKLAMIMLVLEDK	KKKPISSRDGMKLCVETSTTFFDDMWVRQSEKDYQDMLVYLKENDFTKVGE	LTEKNALAMHATT	240			
Q8DR50-28-344	188	YPVETDLKLAMIMLVLEDK	KKKPISSRDGMKLCVETSTTFFDDMWVRQSEKDYONMLVYLKENDFAKVGE	LTEKNALAMHATT	287			
SEQNO 71-317	250	260	270	280	290	300	310	
E1LG71-317	241	KTASPAFSYLTDA	SYEAMDFVRQLREQGEAC	YFTMDAGPNVKVLCQEKDLEHLSEIFGQRYRLIVSKTKDLSQDQCC	317			
E6KKU6-317	241	KTASPAFSYLTDA	SYEAMDFVRQLREQGEAC	YFTMDAGPNVKVLCQEKDLEHLSEIFGQRYRLIVSKTKDLSQDQCC	317			
D4FR73-317	241	KTASPAFSYLTDA	SYEAMDFVRQLREQGEAC	YFTMDAGPNVKVLCQEKDLEHLSEIFGQRYRLIVSKTKDLSQDQCC	317			
E9FW52-28-344	241	KTASPAFSYLTDA	SYEAMDFVRQLREQGEAC	YFTMDAGPNVKVLCQEKDLEHLSEIFGQRYRLIVSKTKDLSQDQCC	317			
Q8DR50-28-344	268	KTASPAFSYLTDA	SYEAMDFVRQLREQGEAC	YFTMDAGPNVKVLCQEKDLEHLSEIFGQRYRLIVSKTKDLSQDQCC	344			

Figure 12

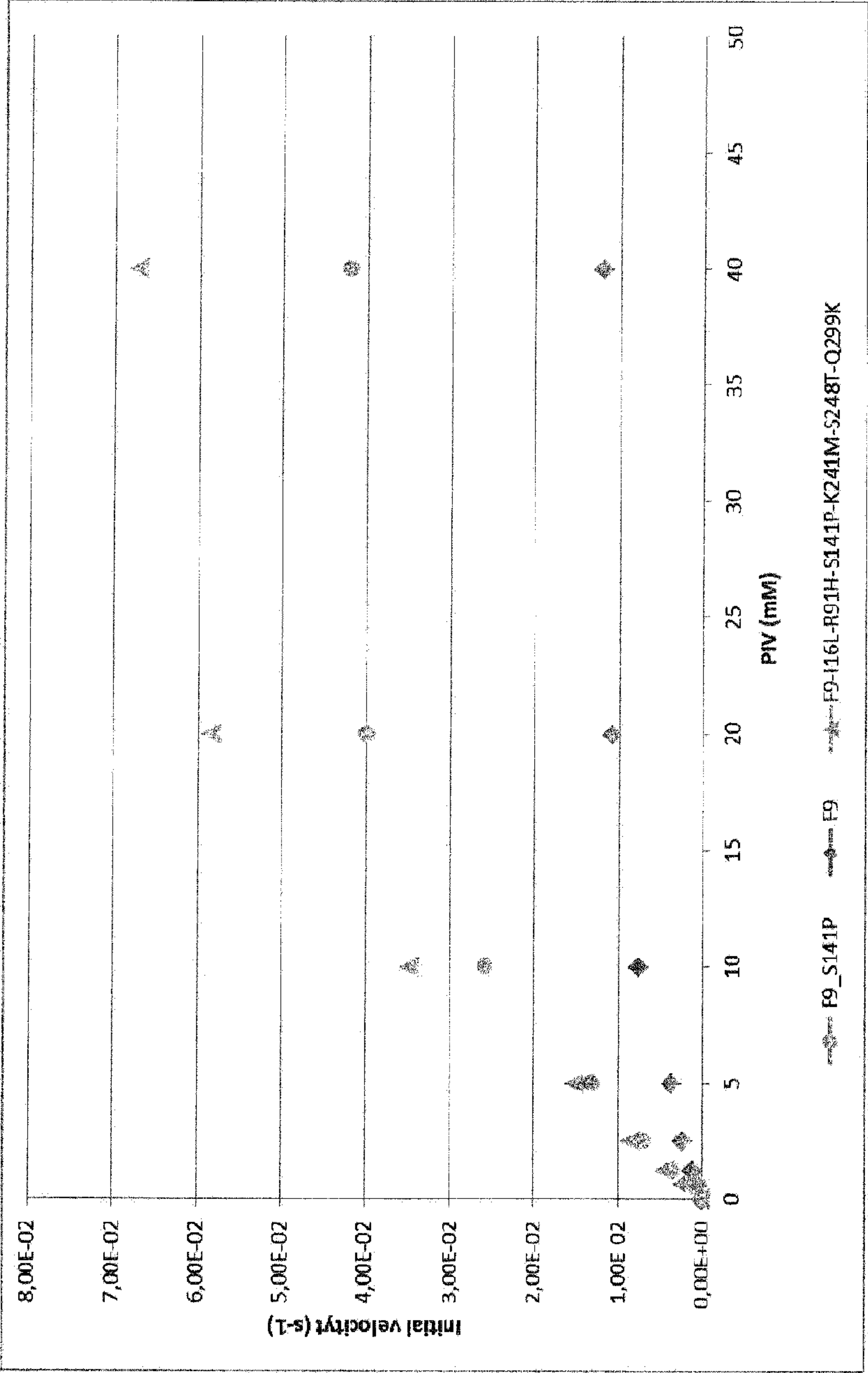


Figure 13

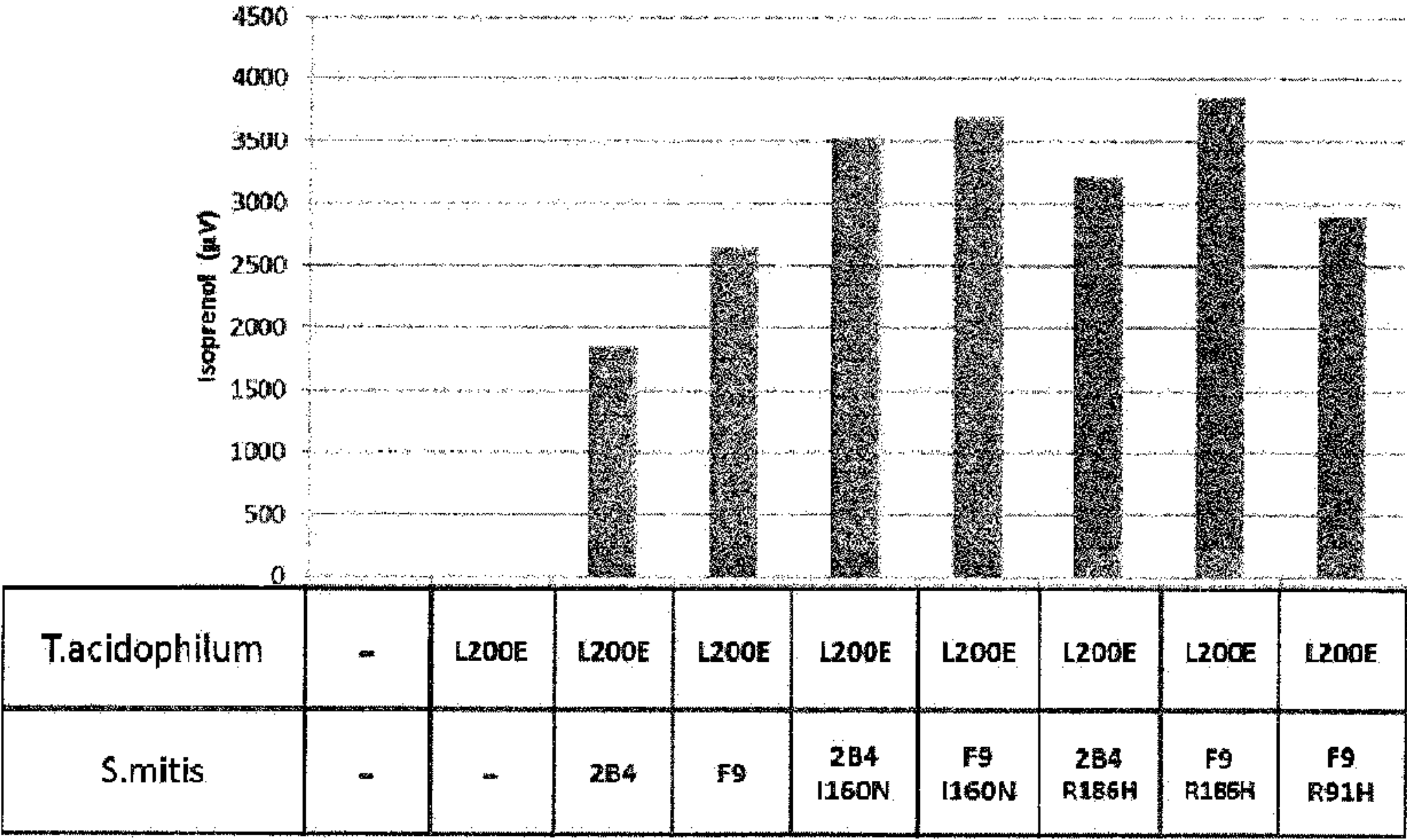


Figure 14

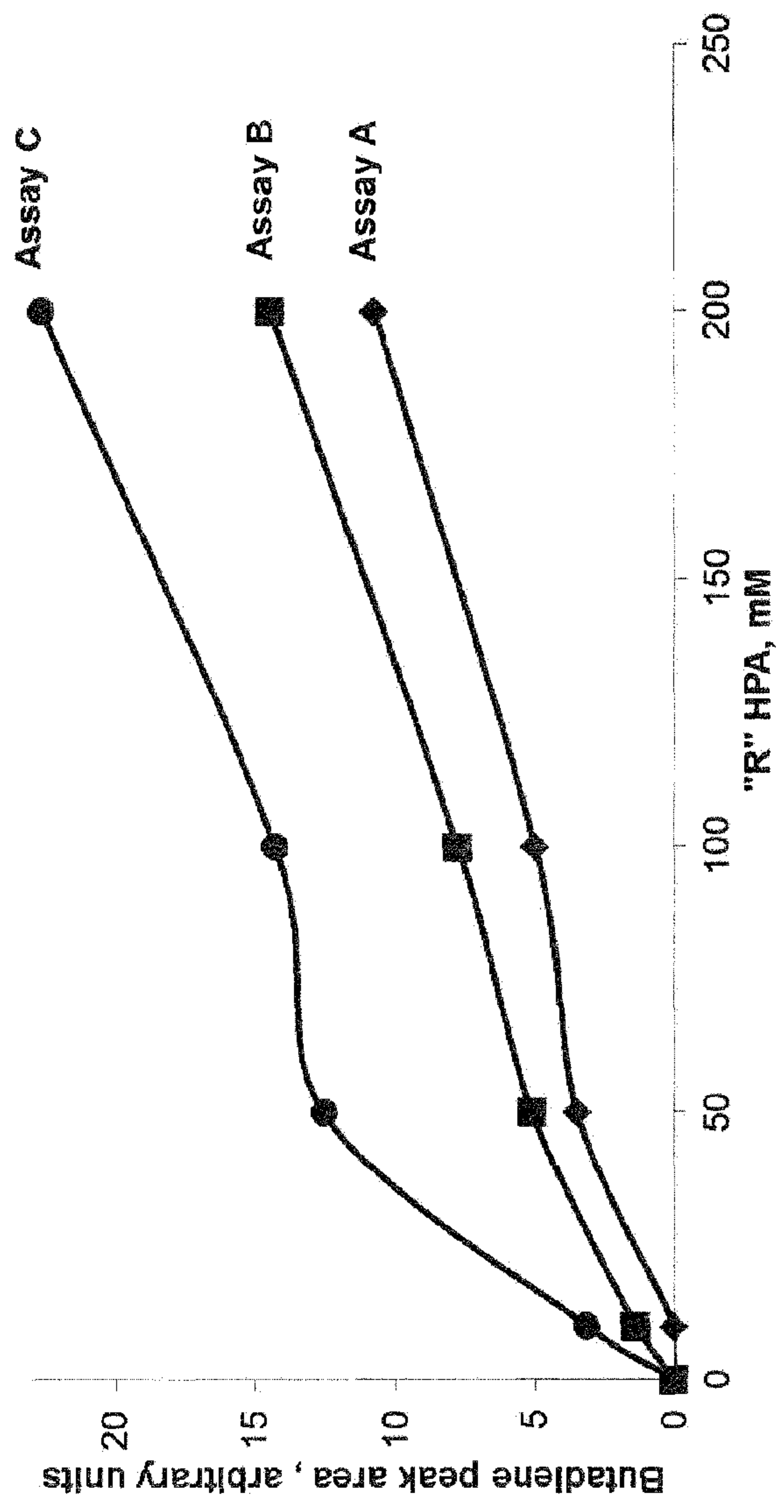


Figure 15

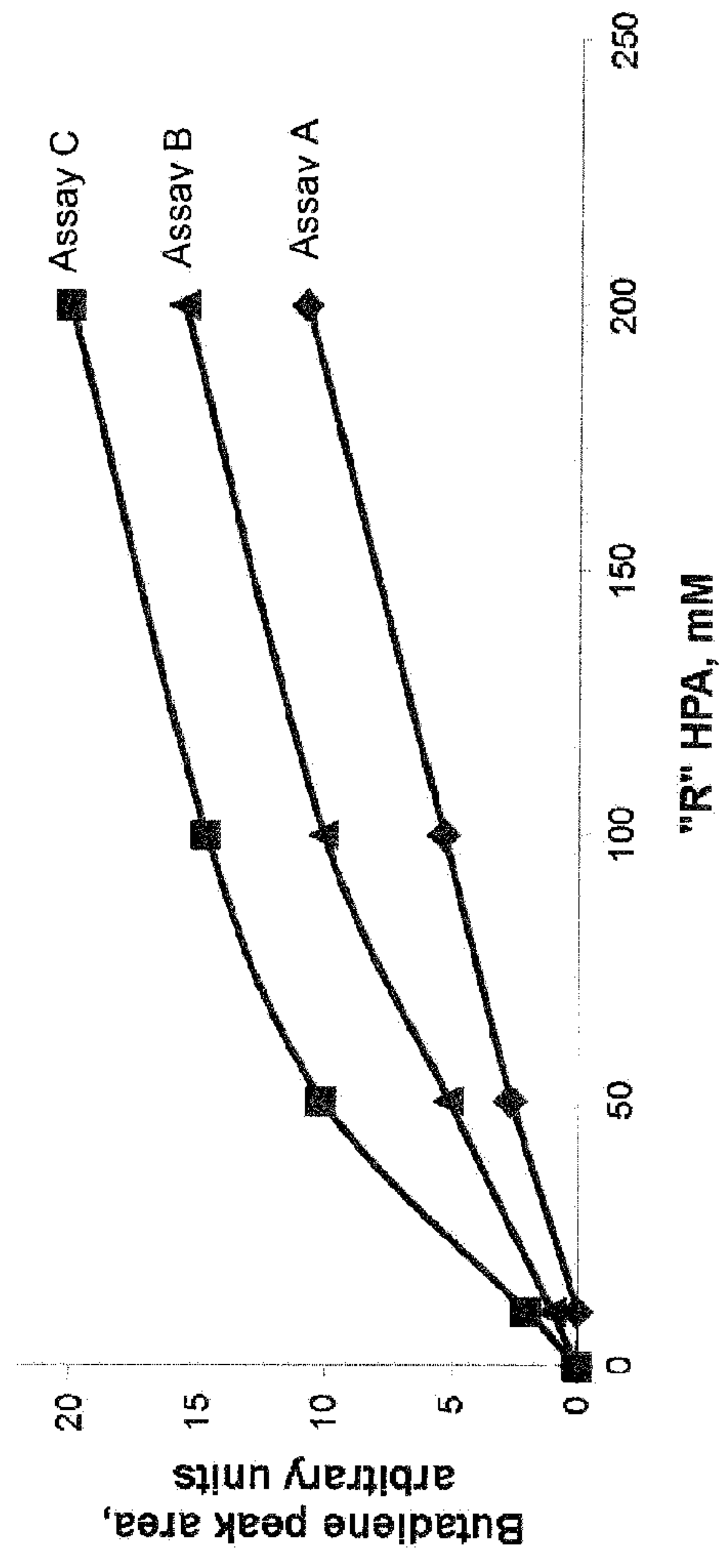


Figure 16