The present invention relates to thermostable puUulanases useful for industrial and scientific purposes. The present invention provides methods for producing the modified puUulanase, enzymatic compositions comprising the modified puUulanase, and methods for use of the enzymatic compositions.
ENZYMES HAVING PULLULANASE ACTIVITY

FIELD OF THE INVENTION

[0001] This invention relates generally to enzymes, polynucleotides encoding the enzymes, the use of such polynucleotides and polypeptides, and more specifically to enzymes having pullulanase activity.

SEQUENCE LISTING

[0002] This application is being filed electronically via the USPTO EFS-WEB server, as authorized and set forth in MPEP § 502.05 and this electronic filing includes an electronically submitted sequence listing; the entire content of this sequence listing is hereby incorporated by reference into the specification of this application. The sequence listing is identified on the electronically filed ASCII (.txt) text file as follows:

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BACKGROUND

[0003] Pullulanase is a specific kind of glucanase, an amylolytic exoenzyme, that degrades pullulan. Type I pullulanases specifically attack alpha-1,6 linkages, while type II pullulanases are also able to hydrolyse alpha-1,4 linkages.

[0004] Pullulanase (EC 3.2.1.41) is also known as pullulan-6-glucanohydrolase (Debranching enzyme). Its substrate, pullulan, is regarded as a chain of maltotriose units linked by alpha-1,6-glycosidic bonds. Pullulanase will hydrolytically cleave pullulan (alpha-glucan polysaccharides).

[0005] Pullulanases are used for several industrial and commercial applications, including, saccharification of starch, liquefaction of starch, production of high-

SUMMARY OF THE INVENTION

[0006] The invention provides an isolated nucleic acid having a sequence as set forth in SEQ ID No.: 1 and variants thereof having at least 50% sequence identity to SEQ ID No.: 1 and encoding polypeptides having pullulanase activity, or encoding polypeptides having pullulanase activity having at least 50% sequence identity to SEQ ID No.: 2.

[0007] One aspect of the invention is an isolated nucleic acid having a sequence as set forth in SEQ ID No.: 1, sequences substantially identical thereto, and sequences complementary thereto.

[0008] Another aspect of the invention is an isolated nucleic acid including at least 10 consecutive bases of a sequence as set forth in SEQ ID No: 1 nucleic acid sequences, sequences substantially identical thereto, and the sequences complementary thereto.

[0009] In yet another aspect, the invention provides an isolated nucleic acid encoding a polypeptide having a sequence as set forth in SEQ ID No.: 2 and variants thereof encoding a polypeptide having pullulanase activity and having at least 50% sequence identity to such sequences. Another aspect of the invention is an isolated nucleic acid encoding a polypeptide or a functional fragment thereof having a sequence as set forth in SEQ ID No.: 2, and sequences substantially identical thereto.
Another aspect of the invention is an isolated nucleic acid encoding a polypeptide having at least 10 consecutive amino acids of a sequence as set forth in SEQ ID No.: 2, and sequences substantially identical thereto.

In yet another aspect, the invention provides a purified polypeptide having a sequence as set forth in SEQ ID No.: 2, and sequences substantially identical thereto.

Another aspect of the invention is a method of making a polypeptide having a sequence as set forth in SEQ ID No.: 2, and sequences substantially identical thereto. The method includes introducing a nucleic acid encoding the polypeptide into a host cell, wherein the nucleic acid is operably linked to a promoter, and culturing the host cell under conditions that allow expression of the nucleic acid.

Another aspect of the invention is a method of making a polypeptide having SEQ ID No.: 2, and sequences substantially identical thereto. The method includes introducing a nucleic acid encoding the polypeptide into a host cell, wherein the nucleic acid is operably linked to a promoter, and culturing the host cell under conditions that allow expression of the nucleic acid, thereby producing the polypeptide.

Another aspect of the invention is a method of generating a variant including obtaining a nucleic acid having a sequence as set forth in SEQ ID No.: 1 or sequences substantially identical thereto, sequences complementary to the sequences of SEQ ID No.: 1, fragments comprising at least 30 consecutive nucleotides of the foregoing sequences, and changing one or more nucleotides in the sequence to another nucleotide, deleting one or more nucleotides in the sequence, or adding one or more nucleotides to the sequence.

Another aspect of the invention is a computer readable medium having stored thereon a sequence as set forth in SEQ ID No.: 1 sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in SEQ ID No.: 2 amino acid sequences, and sequences substantially identical thereto.

Another aspect of the invention is an assay for identifying fragments or variants of SEQ ID No.: 2 amino acid sequences, and sequences substantially identical
thereto, which retain the enzymatic function of the polypeptides of SEQ ID No.: 2 amino acid sequences, and sequences substantially identical thereto. The assay includes contacting the polypeptide of SEQ ID No.: 2 amino acid sequences, sequences substantially identical thereto, or polypeptide fragment or variant with a substrate molecule under conditions which allow the polypeptide fragment or variant to function, and detecting either a decrease in the level of substrate or an increase in the level of the specific reaction product of the reaction between the polypeptide and substrate thereby identifying a fragment or variant of such sequences.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

[0018] Figure 1 is a chromatogram displaying the results of a digestion using the pullulanase of SEQ ID No.: 1 & 2 with a 1% corn starch substrate at 50 degrees Celcius, and as further described in Example 1.

[0019] Figure 2 is a chromatogram displaying the results of a digestion using the pullulanase of SEQ ID No.: 1 & 2 with a 1% pullulan at 50 degrees Celcius, and as further described in Example 1.

[0020] Figure 3 is a chromatogram displaying the results of a digestion using the pullulanase of SEQ ID No.: 1 & 2 with a 1% corn starch substrate at 75 degrees Celcius, and as further described in Example 1.

[0021] Figure 4 is SEQ ID No.: 1, the deoxyribonucleic acid (DNA) sequence of the present invention.
EXAMPLES

Example 1
The pullulanase of the present invention (as embodied by SEQ ID No.: 1 & 2) was tested against 1% solids using an aliquot from a trial cell lysate. The results shown in Figures 1, 2, and 3, show that the pullulanase of the present invention is able to cleave both alpha-1,6 and alpha-1,4 bonds. As displayed in Figure 2, the pullulanase of the present invention (as embodied by SEQ ID No. 1 & 2) was assayed with 1% pullulan at 50 degrees Celsius. As displayed in Figure 1, the pullulanase of the present invention (as embodied by SEQ ID NO 1 & 2) was assayed with 1% corn starch at 50 degrees Celcius. As displayed in Figure 3, the pullulanase of the present invention (as embodied by SEQ ID No. 1 & 2) was assayed with 1% corn starch at 75 degrees Celsius. As indicated in figure 1, 2, and 3, the enzyme is a type II pullulanase, as the reaction products (major peaks) are glucose, maltose, and maltotriose. Additionally, the peak 2 product was confirmed to be maltose and not isomaltose, while the peak 3 product was confirmed to be maltotriose and not panose.

Example 2
The pullulanase of the present invention (as embodied by SEQ ID No. 1 & 2) melting point or thermal denaturation was determined using differential scanning calorimetry. The Tm of the present invention (as embodied by SEQ ID No. 1 & 2) is 84 degrees Celsius.
DETAILED DESCRIPTION

[0025] The present invention relates to a pullulanase enzyme, polynucleotides encoding the enzymes, methods of making and using these polynucleotides and polypeptides. The invention is directed to novel polypeptides having pullulanase activity, nucleic acids encoding them. The polypeptides of the invention can be used in a variety of commercial, medical, and industrial contexts. The polypeptides of the invention can be used as, e.g., an additive for a detergent, for processing foods and for chemical synthesis utilizing a reverse reaction, saccharification of starch, liquefaction of starch, production of high-maltose corn syrup, production of high-fructose corn syrup, starch processing, ethanol production, production of cyclodextrins, and production of low-calorie beer, in the baking industry, as well as dental plaque control.

[0026] In one aspect of the invention the pullulanase is a type Π pullulanase or is capable of cleaving both alpha-1,6 and alpha-1,4 bonds. In another aspect of the invention the pullulanase of the present invention is capable of cleaving both alpha-1,6 and alpha-1,4 of pullulan yielding glucose, maltose, and maltotriose. In a further embodiment of the present invention the pullulanase of the present invention is thermostable and or thermotolerant. In a further embodiment of the invention the pullulanase of the present invention is active at 75 degrees Celsius. In a further embodiment of the invention, the pullulanase of the invention is capable of saccharification of starch at higher temperatures then currently employed, thereby reducing processing times, and increasing yields due to lessening the rate of retrogradation of materials resulting in drops in temperature. In a further embodiment of the present invention the pullulanase is capable of enhancing ethanol or glucose production by digesting limit dextrans that an amylase cannot digest.

[0027] In a further embodiment of the present invention the pullulanase of the present invention is coupled with an amylase enzyme.

[0028] In one aspect, the nucleic acid encodes at least one polypeptide having pullulanase activity.
"Synthetic" nucleic acids (including oligonucleotides), polypeptides or proteins of the invention include those prepared by any chemical synthesis, e.g., as described, below.

The phrases "nucleic acid" or "nucleic acid sequence" includes oligonucleotides, nucleotides, polynucleotides, or to a fragment of any of these, to DNA or RNA (e.g., mRNA, tRNA, rRNA) of genomic, recombinant or synthetic origin which may be single-stranded or double-stranded and may represent a sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin, including, e.g., iRNA such as miRNA or siRNA, ribonucleoproteins (e.g., iRNPs). The term encompasses nucleic acids, i.e., oligonucleotides, containing known analogues of natural nucleotides. The term also encompasses nucleic-acid-like structures with synthetic backbones, see e.g., Mata (1997) Toxicol. Appl. Pharmacol. 144:189-197; Strauss-Soukup (1997) Biochemistry 36:8692-8698; Samstag (1996) Antisense Nucleic Acid Drug Dev 6:153-156.

"Recombinant" polypeptides or proteins refer to polypeptides or proteins produced by recombinant DNA techniques; e.g., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide or protein.

The term "gene" includes a nucleic acid sequence comprising a segment of DNA involved in producing a transcription product (e.g., a message), which in turn is translated to produce a polypeptide chain, or regulates gene transcription, reproduction or stability. Genes can include regions preceding and following the coding region, such as leader and trailer, promoters and enhancers, as well as, where applicable, intervening sequences (introns) between individual coding segments (exons).

The invention provides isolated and recombinant nucleic acids, including expression cassettes such as expression vectors encoding the polypeptides of the invention. The invention provides probes comprising or consisting of nucleic acids of the invention. The invention also includes methods for discovering new pullulanase sequences using the nucleic acids of the invention.
includes methods for inhibiting the expression of pullulanase genes, transcripts and polypeptides using the nucleic acids of the invention.

[0034] The nucleic acids of the invention can be made, isolated and/or manipulated by, e.g., cloning and expression of cDNA libraries, amplification of message or genomic DNA by PCR, and the like. In practicing the methods of the invention, homologous genes can be modified by manipulating a template nucleic acid, as described herein. The invention can be practiced in conjunction with any method or protocol or device known in the art, which are well described in the scientific and patent literature.
WHAT IS CLAIMED IS:

1. An isolated, synthetic, or recombinant nucleic acid encoding a polypeptide having pullulanase activity, selected from the group consisting of:

(a) a nucleic acid encoding a polypeptide having pullulanase activity comprising a nucleic acid sequence having at least 50% sequence identity to SEQ ID NO:1;

(b) a nucleic acid encoding a polypeptide having pullulanase activity comprising a nucleic acid sequence having at least 50% sequence identity to SEQ ID NO:1, or a fragment thereof, wherein the fragment encodes a polypeptide having pullulanase activity;

(c) a nucleic acid sequence encoding a polypeptide having pullulanase activity comprising an amino acid sequence having at least 50% sequence identity to SEQ ID NO:2;

(d) a nucleic acid sequence encoding a polypeptide having pullulanase activity comprising an amino acid sequence having at least 50% sequence identity to SEQ ID NO:2, or a fragment thereof, wherein the fragment has pullulanase activity;

(e) the nucleic acid of (a), (b), (c), or (d) encoding a polypeptide having pullulanase activity but lacking a signal sequence or a carbohydrate binding module;

(f) the nucleic acid of (a), (b), (c), (d) or (e) encoding a polypeptide having pullulanase activity, and further comprising a heterologous sequence;

(g) the nucleic acid sequence of (g), wherein the heterologous sequence comprises a sequence encoding a heterologous signal sequence, carbohydrate binding module, catalytic domain (CD), or a combination thereof, or the heterologous signal sequence, carbohydrate binding module, or catalytic domain (CD) is derived from another pullulanase enzyme, or a non-pullulanase enzyme; or
(h) a nucleic acid sequence fully complementary to (a), (b), (c), (d), (e), (f), or (g).

2. The isolated, synthetic, or recombinant nucleic acid of claim 1, wherein the pullulanase activity comprises the cleavage of both alpha-1,6 and alpha-1,4 bonds.

3. The isolated, synthetic, or recombinant nucleic acid of claim 1, wherein the pullulanase activity comprises type II pullulanase activity.

4. The isolated, synthetic, or recombinant nucleic acid of claim 1, wherein the pullulanase activity is thermostable and/or thermotolerant.

5. An isolated, synthetic, or recombinant polypeptide having pullulanase comprising
(a) an amino acid sequence having at least 50% identity, or complete sequence identity to sequence of SEQ ID No. 2;
(b) having the amino acid sequence encoded by the nucleic acid of claim 1;
(c) the amino acid sequence of (a) or (b), and comprising at least one conservative amino acid residue conservative substitutions;
(d) the amino acid sequence of (a) or (b) or (c) or a fragment thereof with pullulanase activity.

6. The isolated, synthetic, or recombinant polypeptide of claim 5, where the pullulanase activity is thermostable.

7. The isolated, synthetic, or recombinant polypeptide of claim 5 wherein the polypeptide retains an pullulanase activity under conditions comprising a temperature range of between about 37°C to about 84°C, or between about 55°C to about 85°C, or between about 70°C to about 84°C.

8. The isolated, synthetic, or recombinant polypeptide of claim 5, where the pullulanase activity is thermotolerant.
9. The isolated, synthetic, or recombinant nucleic acid of claim 20, wherein the polypeptide retains an pullulanase activity after exposure to a temperature in the range from greater than 37°C to about 84°C, from greater than 55°C to about 84°C.

10. A method of hydrolyzing a starch linkage comprising contacting a substance containing the starch with a polypeptide of claim 5, and sequences substantially identical thereto.

11. A method for liquifying a starch containing composition comprising contacting the starch with a polypeptide of claim 5, and sequences substantially identical thereto.

12. A method for producing a syrup comprising the use of the polypeptide of claim 5, and sequences substantially identical thereto.

13. A method for starch liquefaction comprising contacting said starch with with a polypeptide of claim 5 under conditions sufficient for said liquefaction.

14. A detergent additive comprising with a polypeptide of claim 5.

15. A method for producing a high-maltose or a high-glucose syrup or a mixed syrup comprising use of the polypeptide of claim 5.


17. A method of producing ethanol comprising use of the polypeptide of claim 5, wherein the polypeptide is capable of digesting limit dextrins.

18. The method as in any of claims 10-17 further comprising addition of an pullulanase or a combination thereof.

19. A detergent composition comprising the polypeptide of claim 5.
SEQ ID NO: 2

75°C – 1% corn starch

FIGURE 3
SEQ ID No. 1, Pullulanase, DNA

ATGACCCGCAACGACCCCGCCCGGCGGATTCGTTGTCGCGCCACCGGCAACACGGCGAC
CTCAACTTCGAGCACGACCCCGCCACGTGCTCGGCGGGCGATGGGTCGTTGCGCTTCCAGG
CCGGGAGGGCTGTGGATAGGGCGCCATTGCGTCAGGCGAGGCGAGAACATACCGACGGCGCTTGG
CAGACGGAGCGAGATCTGGCAGGGGTACGCGTCTTCACCGACTACGGCTACCCACCCACGGCACG
CAGAATAAGGCCGCGGTTCGCGCCCCTTGACCGCAATCTCGTGTTGGGCCCTCGGACTGGGAGGAAAGAC
ATCGCTACAGGCTTCCCCGGAACGCTTCTGGAATTGGGACACAAAATTCAACAGGACATTGCGGCCCGAGAAGAC
GAGTACCAGCTTTCAACGAGACCTGGAAAACACGTCTTTCTACCTGCAACTGGAAGGACAGGCCGCTTTCCAGAAG
CCCGGTAGCTGCACTGCTGACCGACATTTTCCTGTGGGCGGACCTAGCCGGGTTCTGGAAGAAAGCTACCCACCTCAG
AGCTTTTGCGGCTAGCTGTGCTTACCCAACCGGCTTCTCGACCTCGTCGGCCCGCCACGGCTCACCCACGAG
CTACTAAACATCTCTCCAAATTTCGGCGACCGAGGCACCCCTTCACGCGAGCGGACCGCAGCCGAGG
GATGCGGCGTAGCTTCCGACCTCAGTCACCAACACAGTGGCTTGGCTTCCTCGCTCAGGCTAAAACGATGGGAGGTTCC
GGCGGCGCTCCAGTACTGGAATGTCAAGATGAACTGGGATGCGTGCCCTCAGGCGACCGCCTCCAGAAG
GACACCTTCTCGGGGCTGGAGCTTACCAACACGCAAACCCGAGGTGAAAAGTGATATCCTGCTGAG
GTCGCGAGCACTGGATGCGCATTCCGCTCCAGGCTTCGCGTGCTACCCACAGGGCATCTGGAACCGGAG
GATTCTACCGGAGACCTGCGCAGGGTGGTGGGTTTAAACCGCATGCTACCATGCGTGCGGACAGATCGTGCGC
CGGGACCCCCAGCTGGCTCAAGGGCGACACGGCGATAGCTGTAACGTACCGCATCGGGCGGAGCATTTGTCT
GCGCTTCGCGGGCGCGCGGCGGGGGTCTCTTCTACTGAGTGGACCGCGGCGCTACTACGATCGCTTACAC
CGATTCCCCGGAGGGCGGTTGCGGCGGGTGGTGAACCTTATGTTGCATCAACTCTACCGGTATTCCAGA
TTGGTGGGGGTGGCTCTAGCGACCCCGGTGGCCGACGTTGCGGCGGCTCGGCTGCGTGGCCATGCTTTGCT
CTACGCCCCTCCGGAAATCGGCTAATTTTGACTCGAGGGGAATTAGTGCGTGTTTCTACGGGAGGGCGGCTAGCC
GGTCAAAGCTATACCGCTACAATCAGTGGGGAATGTTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
CGGCAAGGGCTGGGCTGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
TGGCTTTCTCGGGGGCGAGCCAGCGTCAGGAGGTGGTGTTTGGCGGCGCTGTTTCCAAAGGGGCGGCGGAGACCGCA
CTGGCCCTCTCCGGGCGCAGCTGGCGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
ATTGGTTTTCGTATCTCGTACGGGCTGGCGAGTAG
SEQ ID No. 2, Pullulanase, Polypeptide

MTAQRPPPTGPVGVGPTRPQSPGDLNFEDPKLPFSLADGLSVRFQAGEGSVKWAIVQAEGRNYPMHRLWQ
DGSEIWRVALPPSIEKYRIRLETAQNKPAGTPFDANHPVLGLDWGVGSIGYQVFPERFWNGDKTNDMRALETDEYRF
NETWNQNPQATKPYLSNWNPAGELHCUHQYFGDLAGFLKPHLEALGVRLYFNPLFDGSAAHGYDTHYKIS
PKFGEALLRQVLDAAHAQGMRFVIFDFVPNHTGLGFAFQDVKKNGRASQYWNYTIRRWPFPRPGDASYYDTSQG
VGSLPKLNTANPEVKKYLLEVEAHEWMRFGFDGLRVDYPQGIVNREDFYRDLRRVVKGVKPDAYIVAEIWARDPSWLQ
GDQADSLMNAYAIGRDIVLFARCGGGVALYSRRLADLVRITYDPEAVVGQGWNLIGSHDTPRVLTDLGALGDT
SPESLARLRLAMGLLYALPGMPFFQGDECGTGEAGQYPVNELYRYPQWDRCNPDVEFLYQRLGKVRAGLAALQGP
AFRAYAGEGAVLAFRGLRGEPGQEVLA</doc>

FIGURE 5
A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 9/44, C12N 9/26, C07K 1/00, C07K 14/00 (2014.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 435/210, 435/201, 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGBP,USPT,USOC,EPAB,JPAB); PatBase, Google/Scholar: Pullulanase, pullulan-6-glucanohydrolase, Debranching enzyme
GenCore 6.4.1 : SEQ ID NO:1, 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category</th>
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<th>Relevant to claim No.</th>
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<td>A</td>
<td>Hii, et al. Pullulanase: role in starch hydrolysis and potential industrial applications. Enzyme Res. 2012, 2012:921362; Abstract; pg 4, col 2, 1st full para; pg 6, Table 2</td>
<td>1-4</td>
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* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "&" document member of the same patent family

Date of the actual completion of the international search: 23 May 2014 (23.05.2014)

Date of mailing of the international search report: 05 AUG 2014

Authorized officer: Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

P.O. Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Further documents are listed in the continuation of Box C.
Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:  
   because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:  
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: claims 1-4, drawn to an isolated, synthetic, or recombinant nucleic acid encoding a polypeptide having pullulanase activity, wherein said nucleic acid is at least 50% sequence identical to SEQ ID NO: 1 or encodes a polypeptide having pullulanase activity comprising an amino acid sequence at least 50% sequence identical to SEQ ID NO:2.

Group II: claims 5-19, drawn to an isolated, synthetic, or recombinant polypeptide having pullulanase comprising an amino acid sequence having at least 50% identity to sequence of SEQ ID NO: 2

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-4

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)
In continuation of Box III. Observations where unity of invention is lacking:

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features
The special technical feature of each invention of Group I is a nucleic acid of a specified nucleic acid sequence.
The special technical feature of each invention of Group II is a protein a specified amino acid sequence.

Common Technical Features
The inventions of Groups I-II share the technical feature of a polypeptide having pullulanase activity and comprising an amino acid sequence at least 50% identical to sequence of SEQ ID NO. 2. However, this shared technical feature does not represent a contribution over prior art as being anticipated by NCBI Reference Sequence: YP_003684815.1 titled "alpha amylase (Meiothermus silvanus DSM 9946)? (23 December 2012), [Retrieved from the Internet 18 May 2014: <http://www.ncbi.nlm.nih.gov/protein/297565843?sat=17&satkey=24217796>] (hereinafter "NCBI") that discloses an amino acid sequence of alpha amylase from Meiothermus silvanus DSM 9946, said sequence is 98.2% identical with the claimed SEQ ID NO: 2. NCBI further discloses that said alpha amylase possesses pullulanase activity (CDS: 1..703... KEGG: tga: TGAM_1752 pullulan hydrolase type III (pulhA); PFAM: alpha amylase catalytic region; alpha amylase all-beta...”). As said technical feature was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Groups I-II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.