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(72) Inventeurs/Inventors:
MILLER, BRIAN S., US;
SHETTY, JAYARAMA K., US

(73) Propriétaire/Owner:
GENENCOR INTERNATIONAL, INC., US

(74) Agent: SMART & BIGGAR

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The present invention relates to modified pullulanases useful in the starch industry. The present invention provides methods for producing the modified pullulanase, enzymatic compositions comprising the modified pullulanase, and methods for the saccharification of starch comprising the use of the enzymatic compositions.

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(71) Applicant: GENENCOR INTERNATIONAL, INC. [US/US]; 4 Cambridge Place, 1870 South Winton Road, Rochester, NY 14618 (US).

(72) Inventors: MILLER, Brian, S.; 1446 Floribunda Avenue, Burlingame, CA 94010 (US). SHETTY, Jayarama, K.; 4806 Braxton Place, Pleasanton, CA 94566 (US).

(74) Agent: GLAISTER, Debra, J.; Genencor International, Inc., 925 Page Mill Road, Palo Alto, CA 94304–1013 (US).

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(57) Abstract

The present invention relates to modified pullulanases useful in the starch industry. The present invention provides methods for producing the modified pullulanase, enzymatic compositions comprising the modified pullulanase, and methods for the saccharification of starch comprising the use of the enzymatic compositions.

MODIFIED FORMS OF PULLULANASE

Field of the Invention

The present invention relates to modified forms of pullulanase which maintain the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond, compositions which comprise the modified pullulanase, methods of making the modified pullulanase and methods of using the modified pullulanase, especially for the saccharification of starch.

Background of the Invention

Starch, the essential constituents of which are linear amylose and branched amylopectin glucose polymers can be converted into simple sugars by an enzymatic process carried out in two stages: one stage of liquefaction of the starch and one stage of saccharification of the liquefied starch. In order to obtain a high conversion level of the starch, pullulanase (E.C. 3.2.1.41, α-dextrin 6-glucano-hydrolase also termed alpha-1,6-glucosidase) has been used to catalyse the hydrolysis of alpha-1,6-glucosidic bonds.

Pullulanase enzymes in the art include those known to have optimum activity at acidic pH as well as those known to have activity at alkaline pH. Pullulanases described in the art include pullulanase derived from a strain of *Bacillus acidopullulyticus* described as having an optimum activity at a pH of 4-5 at 60 ° C (United States Patent No. 4,560,651); pullulanase derived from *Bacillus naganoensis* described as having a maximum activity at a pH of about 5, measured at 60 ° C and a maximum activity at a temperature of about 62.5 ° C, measured at a pH of 4.5 (United States Patent No. 5,055,403); pullulanase derived from *Bacillus sectorramus* described as having an optimum pH at 5.0 to 5.5 and an optimum temperature at 50 ° C (United States Patent No. 4,902,622); and pullulanase derived from *Bacillus brevis* PL-1 described as having activity at 4.5-5.5 at 60 ° C (JP 04/023985).

Pullulanase can be used with glucoamylase or ß-amylase for the production of high glucose and high maltose syrups. In addition to increasing the yields of sugars, pullulanase reduces reaction time, allows high substrate concentrations and a reduction of up to 50% in the use of glucoamylase (Bakshi et al. (1992) Biotechnology Letters vol.14 pp.689-694).

Summary of the Invention

The present invention relates to the surprising and unexpected discovery by Applicants that modified forms of pullulanase retain the ability to catalyze the

hydrolysis of an alpha-1,6-glucosidic bond. The present invention provides modified forms of pullulanase and methods for producing the modified pullulanase, especially in recombinant host microorganisms. The present invention further relates to enzymatic compositions comprising a modified form of pullulanase useful in the saccharification of starch and methods for the saccharification of starch comprising the use of the enzymatic compositions.

The present invention is based, in part, upon the discovery that when pullulanase obtained from Bacillus deramificans was recombinantly expressed and cultured in Bacillus licheniformis, the pullulanase produced was a mixture of modified forms yet the modified forms of pullulanase surprisingly retained the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. The modified forms comprised B.deramificans pullulanase truncated at the amino terminus, i.e., having a deletion of amino acids from the amino terminus, and B.deramificans having additional amino acids at the amino terminus of the mature pullulanase. Therefore, in one aspect, the present invention provides modified pullulanase having a deletion of amino acids from the amino terminus of a pullulanase obtainable from a gram-positive or a gram-negative microorganism as long as the modified pullulanase retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. In another aspect, the present invention provides modified pullulanase having additional amino acids at the amino terminus of a pullulanase obtainable from a gram-negative or gram positive microorganism as long as the modified pullulanase retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. The present invention also encompasses amino acid variations of a pullulanase obtainable from a gram-negative or gram positive microorganism as long as the modified pullulanase retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond.

In one embodiment, the modified pullulanase is a modification of pullulanase obtainable from Klebsiella species. In another embodiment, the modified pullulanase is a modification of pullulanase obtainable from Bacillus species. In yet another embodiment, the modified pullulanase is a modification of pullulanase obtainable from Bacillus including but not limited to *B.subtilis, B. deramificans, B. stearothermophilus, B. naganoensis, B. flavocaldarius, B. acidopullulyticus, Bacillus sp APC-9603, B. sectorramus, B. cereus, B. fermus*. In a preferred embodiment, the

modified pullulanase is a modification of pullulanase obtainable from *B. deramificans* having the designation T89.117D (LMG P-13056) deposited June 21,1993 under the Budapest Treaty in the Belgian Coordinated Collections of Microorganisms, LMG culture collection, University of Ghent, Laboratory of Microbiology, K.L. Ledeganckstraat 35, B-9000 GHENT, Belgium.

In one embodiment, the modified pullulanase has a deletion of about 100 amino acids from the amino terminus of a pullulanase. In another embodiment, the modified pullulanase has a deletion of about 200 amino acids from the amino terminus of a pullulanase and in yet another embodiment, the modified pullulanase has a deletion of about 300 amino acids from the amino terminus of a pullulanase.

In a further embodiment, the modified pullulanase has a deletion of 98 amino acids from the amino terminus of pullulanase obtainable from B. deramificans. In an additional embodiment, the modified pullulanase has a deletion of about 102 amino acids from the amino terminus of pullulanase obtainable from B. deramificans. In a further embodiment, the modified pullulanase has at least one additional amino acid at the amino terminus of pullulanase obtainable from B. deramificans. In another embodiment, the modified pullulanase has an additional amino acid residue, Alanine, added to the amino terminus of pullulanase obtainable from B. deramificans.

In a preferred embodiment, the present invention provides a truncated pullulanase which has the amino acid sequence of SEQ ID NO:2 (Figure 1), wherein the N-terminus of said truncated pullulanase is amino acid residue 99 or amino acid residue 103 of SEQ ID NO: 2, and wherein said truncated pullulanase is capable of catalyzing the hydrolysis of an alpha-1,6-glucosidic bond. Also provided is a nucleic acid comprising a polynucleotide sequence encoding the truncated pullulanase described herein, an expression vector comprising the nucleic acid and a host microorganism comprising the expression vector.

Modified forms of pullulanase having a decrease in molecular weight provide the advantage of higher specific activity (activity/unit weight) and

therefore, less weight of pullulanase activity is required in a saccharification process to obtain results equivalent to the use of a naturally occurring pullulanase obtainable from or produced by a microorganism. The recombinant production of modified pullulanase as taught herein provides for enzymatic compositions comprising at least 60% and at least 80% pullulanase activity. In one embodiment, the enzymatic composition comprises at least one modified pullulanase. In another embodiment, the enzymatic composition comprises more than one modified pullulanase. Such enzymatic compositions are advantageous to the starch processing industry due to their ability to produce a high glucose yield over a shortened saccharification time without the loss of glucose yield associated with reversion reaction products. Furthermore, it was unexpectedly found that in using an enzymatic composition comprising 20% glucoamylase and 80% pullulanase, higher starting dissolved solids (DS) could be used in a saccharification process, thereby increasing production plant capacity without an increase in capital investment. Additionally, saccharification at higher

dissolved solids increases mechanical compression capacity thereby providing for a more energy efficient process.

In one embodiment, the present invention provides modified pullulanase produced by the method comprising the steps of obtaining a recombinant host cell comprising nucleic acid encoding mature pullulanase, culturing said host cell under conditions suitable for the production of modified pullulanase and optionally recovering the modified pullulanase. In one embodiment, the host cell is Bacillus, including but not limited to *B.subtilis*, *B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus and Bacillus thuringiensis*. In a preferred embodiment, the Bacillus cell is B. licheniformis which comprises a first gene encoding the Carlsberg protease and a second gene encoding endo Glu C, the first and/or second gene which codes for the protease(s) having been altered in the Bacillus species such that the protease activity is essentially eliminated and the nucleic acid encoding the mature pullulanase is obtainable from B. deramificans.

In an alternative embodiment, the present invention provides methods for the production of a modified pullulanase in a recombinant host cell comprising the steps of obtaining a recombinant microorganism comprising nucleic acid encoding a modified pullulanase, culturing the microorganism under conditions suitable for the production of the modified pullulanase and optionally recovering the modified pullulanase produced. In one embodiment, the host cell is a gram-negative or gram-positive microorganism. In another embodiment, the host cell is a Bacillus including but not limited to *B.subtilis*, *B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus and Bacillus thuringiensis*. In another embodiment, the Bacillus cell is B. licheniformis which comprises a first gene encoding the Carlsberg protease and a second gene encoding endo Glu C, the first and/or second gene which codes for the protease(s) having been altered in the Bacillus species such that the protease activity is essentially eliminated and the nucleic acid encodes a modified pullulanase that is a modification of pullulanase obtainable from B.deramificans.

The present invention also provides nucleic acid comprising a polynucleotide sequence encoding modified pullulanase. In one embodiment, the nucleic acid has at least 70% identity, at least 80% identity, at least 90% identity or at least 95%

identity to the polynucleotide sequence shown in SEQ ID NO: 1, which encodes pullulanase obtainable from B. deramificans. The present invention also provides expression vectors and host microorganisms comprising nucleic acid encoding a modified pullulanase of the present invention.

The present invention provides an enzymatic composition comprising at least one modified pullulanase of the present invention. In one embodiment, the enzymatic composition comprises multiple modified pullulanase forms. In another embodiment, the composition further comprises an enzyme selected from the group consisting of glucoamylase, alpha-amylase, beta-amylase, alpha-glucosidase, isoamylase, cyclomaltodextrin, glucotransferase, beta-glucanase, glucose isomerase, saccharifying enzymes, and/or enzymes which cleave glucosidic bonds. In a preferred embodiment, the enzymatic composition comprises a modified pullulanase and glucoamylase. In one embodiment, the glucoamylase is derived from an Aspergillus strain. In another embodiment, the glucoamylase is derived from an Aspergillus strain including but not limited to Aspergillus niger, Aspergillus awamori and Aspergillus foetidus. The enzymatic composition may be in a solid form or a liquid form. In one embodiment of the present invention, the enzymatic composition comprises at least 60% modified pullulanase and in another embodiment, the composition comprises at least 80% modified pullulanase.

The present invention also provides a process for the saccharification of starch, wherein said process allows for reduced concentrations of saccharification reversion by-products, comprising the step of contacting aqueous liquified starch with an enzyme composition comprising modified pullulanase. In one embodiment, the process further comprises the steps of heating said liquified starch, and recovering product. In one embodiment of the process, the enzyme composition further comprises glucoamylase. In another embodiment of the process, the contacting is at a pH of about less than or equal to 7.0 and greater than or equal to 3 and in yet another, the pH is about 4.2. In a further embodiment of the process said heating is at a temperature range of between 55 and 65 degrees C. In another embodiment, the temperature is about 60 degrees C.

Brief Description of the Drawings

Figures 1A-1G illustrate the nucleic acid (SEQ ID NO:1) encoding the mature amino acid (SEQ ID NO:2) sequence of pullulanase obtainable from B. deramificans.

Figures 2A-2G are an alignment of amino acid sequences of pullulanase obtainable from B. deramificans (designated pullseqsig.seq.PRO), B. subtilis (designated subpull.seq.pro), and K. pneumonia (designated klebpnseqsig.seq.pro) showing the conserved domains and variability of the amino terminus of these pullulanases. This alignment also includes the signal sequences for the respective pullulanases.

Figures 3A-3C illustrate a timecourse of fermentation and the various species of modified pullulanase that are formed during the fermentation. Peak 1 designates the mature B. deramificans pullulanase having a molecular weight of 105 kD; peak 2 designates the modified pullulanase which has a deletion of 102 amino acids from the amino terminus of mature B. deramificans pullulanase; and peak 3 designates the modified pullulanase which has a deletion of 98 amino acids from the amino terminus as measured by standard HPLC analysis. Figure 3A illustrates the fermentation over 37 hours. Figure 3B illustrates the fermentation over 60 hours. Figure 3C illustrates the fermentation over 70 hours.

Figures 4A-4D illustrate the stability of the modified pullulanase species as a function of pH as measured by standard HPLC analysis. Figure 4A illustrates the pullulanase stability at 24 hours at a pH of 4.5 at room temperature. Figure 4B illustrates the pullulanase stability at 24 hours at a pH of 5.5 at room temperature. Figure 4C illustrates the pullulanase stability at 24 hours at a pH of 6.5 at room temperature. Figure 4D illustrates the pullulanase stability at 96 hours at a pH of 4.5 at room temperature.

Figures 5A-5C illustrate the effect of enzymatic compositions comprising various pullulanase and glucoamylase concentrations on the final glucose yield and disaccharide formation over saccharification time. The solid line refers to an enzymatic blend comprising 80% pullulanase activity (including modified pullulanase having a deletion of 98 amino acids from the amino terminus of B. deramificans; modified pullulanase having a deletion of 102 amino acids from the amino terminus of B. deramificans; mature B. deramificans pullulanase and mature B. deramificans pullulanase having an additional amino acid (alanine) on the amino terminus) and 20% glucoamylase (20:80). The dotted line refers to an enzymatic composition comprising an enzyme blend comprising 75% glucoamylase obtainable from Aspergillus sp. and 25% mature pullulanase obtainable from B. deramificans (75:25). The solid line with squares refers to di-saccharides formed with the enzyme blend

comprising 20% glucoamylase and 80% pullulanase activity as described above (20:80) over the saccharification time and the dotted line with circles refers to the di-saccharides formed with the 75:25 over the saccharification time. The left X-axis is % glucose yield and the right X-axis is % di-saccharides. Figure 5A refers to the saccharification process using 0.550 liters of enzymatic composition per metric ton of dissolved solids; Figure 5B refers to the saccharification process using 0.635 liters of enzymatic composition per metric ton of dissolved solids; Figure 5C refers to the saccharification process using 0.718 liters of enzymatic composition per metric ton of dissolved solids. This Figure illustrates that a 20:80 enzymatic composition is able to increase the final glucose yield without an increase in undesirable disaccharide formation.

Figure 6 illustrates the effect of dissolved solids (%w/w) (Yaxis) on the final glucose yield during saccharification of liquefied starch using enzyme compositions 20:80, 75:25, and 100 % glucoamylase at 0.55 liters of enzyme per metric ton of dissolved solids. Line A is the enzymatic composition 20:80 described in Figures 5A-5C; line B is the enzymatic composition 75:25 and line C is an enzymatic composition comprising 100% glucoamylase.

Detailed Description

Definitions

The term pullulanase as used herein refers to any enzyme having the ability to cleave the alpha-1,6 glucoside bond in starch to produce straight chain amyloses. These enzymes are preferably classified in EC 3.2.1.41 and include neopullulanases.

As shown in Figures 2A-2D, there are regions of similarity among pullulanases obtainable from gram positive and gram negative microorganisms. The amino acid alignment of pullulanase obtainable from Bacillus deramificans with pullulanase obtainable from K. pneumonia and B. subtilis reveals that when the conserved domains are aligned, the amino terminus not associated with the conserved domains is of varying length. As used herein, the term "modified" when referring to pullulanase means a pullulanase enzyme in which the conserved domains are retained while any length of amino acids in the amino terminus portion of the naturally occurring amino acid sequence not associated with the conserved domains has been altered by a deletion of these amino acid residues or by addition of at least one amino acid to the amino terminus as long as the modified pullulanase retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. The

deletion in the amino terminal amino acids of a pullulanase can be of varying length, but is at least three amino acids in length and the deletion can go no further than the beginning of the first conserved domain which in B. deramificans is the tyrosine at amino acid residue 310 as shown in Figures 1A-1E. In one embodiment, the deletion is about 100 amino acids from the amino terminus of the mature pullulanase. In another embodiment, the deletion is about 200 amino acids from the amino terminus of the mature pullulanase and in another embodiment, the deletion is about 300 amino acids from the amino terminus of the mature pullulanase. In a preferred embodiment, the modification is a deletion of 98 amino acids from the amino terminus of B. deramificans. In yet another embodiment, the deletion is 102 amino acids from the amino terminus of B. deramificans. In a further embodiment, the modification is an addition of at least one amino acid to the amino terminus of the naturally occurring mature pullulanase obtainable from Bacillus deramificans. In another preferred embodiment, the amino acid residue, Alanine, is added to the amino terminus of the mature pullulanase. As used herein the term "mature" refers to a protein which includes the N-terminal amino acid residue found after the natural cleavage site of the signal sequence.

As illustrated in Figures 2A-2D, B. deramificans pullulanase and K. pneumonia pullulanase are examples of pullulanases having similarities in the length of the amino terminus up to the beginning of the first conserved domain (which in B.deramificans is amino acid residue 310 Tyrosine). B. subtilis pullulanase is an example of a pullulanase having a shorter length of amino acid residues up to the beginning of the first conserved domain as shown in Figure 2B.

As used herein, "nucleic acid" refers to a nucleotide or polynucleotide sequence, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be double-stranded or single-stranded, whether representing the sense or antisense strand. As used herein "amino acid" refers to peptide or protein sequences or portions thereof. The present invention encompasses polynucleotides having at least 70%, at least 80%, at least 90% and at least 95% identity to the polynucleotide encoding *B. deramificans* pullulanase, as well as polynucleotides encoding a pullulanase activity capable of hybridizing to nucleic acid encoding *B. deramificans* pullulanase under conditions of high stringency.

The terms "isolated" or "purified" as used herein refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

Detailed Description of the Preferred Embodiments

The present invention relates to the discovery that pullulanase recombinantly produced in a Bacillus host is modified yet unexpectedly retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. The modification of the pullulanase product recombinantly produced appears to be a result of misprocessing of the signal sequence by a signal peptidase as well as susceptibility to extracellular proteolytic processing. The modified pullulanase is used to produce compositions and methods useful in the starch industry.

I. Pullulanase Sequences

The present invention encompasses any modified pullulanase which retains the ability to catalyze the hydrolysis of an alpha-1,6-glucosidic bond. A variety of pullulanases have been described in the art, including those obtainable from or naturally produced by gram-positive microorganisms as well as gram-negative microorganisms. Microorganisms which naturally produce pullulanase include, but are not limited to, *B.deramificans* (having the designation T89.117D in the LMG culture collection, University of Ghent, Laboratory of Microbiology-K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium) the nucleic acid (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequence being disclosed in Figures 1A-1E; *B.naganoensis* (American Type Culture Collection, ATCC accession number 53909), disclosed in United States Patent No. 5,056,403 issued October 8, 1991; *B. acidopullulyticus* (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, NCIB 11607, NCIB 11610, NCIB 11611, NCIB 11636, NCIB 11637, NCIB 11639, NCIB 11638, NCIB 11647, NCIB 11777), disclosed in United States Patent No. 4,560,651, issued December 24, 1985; *B. sectorramus* (Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Yatabe-machi, Tsukuba-gun, Ibaraki 305 Japan FERM BP-1471), disclosed in United States Patent No. 4,902,622, issued February 20, 1998; *Bacillus* FERM BP-4204 disclosed in United States Patent No. 5,387,516 issued February 7, 1995; *B. stearothermophilus* (SWISS-PROT id NEPU_BACST ac P38940); *B. cereus* var. *mycoides* (IFO 300) described in Y.Takasaki et al., 1976, Agric. Biol. Chem. 40:1515; *B. fermus* (IFO 3330); *Klebsiella pneumonia*, United States Patent No.

3,897,305 (SWISS-PROT id PULA_KLEPN ac P07206 and ATCC 15050; *Klebsiella aerogenes* (SWISS-PROT id PULA_KLEAE ac P07811); *Thermoanaerobium brockii* (ATCC No. 33075), United States Patent No. 4,628,028; *Streptomyces sp.* described in M. Yagisawa et al., 1972, J. Ferment. Technolo. 50:572; Caldicellulosiruptor saccharolyticus disclosed in Albertson et al., 1997, Biochimica et Biophysica Acta 1354:35-39; *Eschericia intermedia* Ueda et al., 1967, Applied Microbiology vol 15:492 United States Patent No. 3,716,455 (issued 1973) *Streptococcus mites* Walker 1968, Biochem. J., vol. 108:33; *Streptomyces* (Ueda et al., 1971, J. Ferment. Tech. Vol. 49: 552); *Flavochromogenes,* as described in United States Patent No. 4,902,622; *Flavobacterium esteromaticum* Japanese Patent Application Kokoku 18826/1973; *Cytophaga* United States Patent No. 3,790,446 issued 1974; *Lactobacillus*, *Micrococcus*, *Nocardia*, *Staphylococcus*, *Azotobactger*, *Sarcina* England patent 11260418, United States Patent No. 3,827940 issued 1974; and *Actinomycetes* United States Patent No. 3,741,873 issued 1973. Any pullulanase known in the art which comprises the conserved pullulanase regions as shown in Figures 2A-2D can be modified to have deletions or additions to the amino terminus as long as the modified pullulanase retains the ability to catalyse the hydrolysis of an alpha-1,6-glucosidic bond.

A nucleic acid sequence encoding a pullulanase can be obtained from a microorganism through hybridization technology using the nucleic acid sequences that encode the conserved domains of pullulanases (as shown in Figures 2A-2D) as primers and/or probes. (United States Patent 5,514,576; Southern, E. 1979, Methods Enzymol. 68:152-176; Saiki, et al. 1988, Science 239:487-491). In one embodiment disclosed herein for *B.deramificans* pullulanase, the naturally occurring nucleic acid (SEQ ID NO:1) encoding a mature pullulanase was introduced into *B.licheniformis* having a deletion of the Carlsburg protease (Jacobs et al., 1985, Nucleic Acid Research 13:8913-8926) and endoGluC proteases (Kakudo et al., 1992, Journal of Bio. Chem. Vol. 267:23782-23788), the B.licheniformis comprising the nucleic acid encoding the mature pullulanase was cultured under conditions suitable for expression of said nucleic acid and secretion of the expressed pullulanase. The protease deletions in B.licheniformis were made through techniques known to those of skill in the art. Through the fermentation process, the expressed pullulanase was cleaved extracellularly into multiple pullulanase species which retain the ability to

catalyse the hydrolysis of alpha-1,6-glucosidic bonds. The multiple species are a pullulanase having a deletion of the first 98 amino acid residues from the amino terminus and starting at glutamic acid, a pullulanase having a deletion of the first 102 amino acid residues from the amino terminus (and starting at glutamic acid), and a pullulanase having the addition of at least one amino acid residue to the amino terminus of the mature pullulanase, along with the mature pullulanase as shown in Figures 1A-1E. As shown in Example II, it appears that the extracellular cleaving into multiple species may be due to a protease activity in the fermentation broth.

In an alternate embodiment of the present invention, nucleic acid encoding a mature pullulanase is genetically engineered to create a modified pullulanase having a deletion of amino acids at the amino terminus or having amino acids added at the amino terminus. The genetically engineered pullulanase is introduced into a host cell, preferably a Bacillus host cell, and cultured under conditions suitable for expression and secretion of the modified pullulanase. Nucleic acid encoding a mature pullulanase can be a naturally occurring sequence, a variant form of the nucleic acid or from any source, whether natural, synthetic, or recombinant.

Regional sequence homologies in starch degrading enzymes have been disclosed in Janse et al. (1993) Curr. Genet. 24:400-407. Janse disclose the conserved regions in α-amylases that are implicated in substrate binding, catalysis, and calcium binding. An amino acid alignment of B.deramificans, B.subtilis and K. pneumonia pullulanases is shown in Figures 2A-2D.

When homologies were compared in starch degrading enzymes by Janse et al., four conserved regions where noted, Regions 1, 2, 3, and 4. Three of these regions were associated with specific functions found in starch-degrading enzymes: region 1: DVVINH; region 2: GFRLDAAKH; and region 4: FVDVHD. Further analysis of five Type I pullulanase sequences by Albertson et al.(1997, Biochimica et Biophysica Acta 1354:35-39) revealed other conserved regions among a group of gram-positive and gram-negative pullulanases. These include regions called DPY, A, B, C, D, E, and YNWGY. Two regions, DPY and YNWGY were identified as being characteristic of true pullulanases. Conserved regions A-E align closely with β-sheet elements as defined for amylases. In addition, two other conserved regions closer to the N-terminus of the pullulanase, referred to as Y and VWAP in Figures 2A-2D, indicate the limits of amino acid truncations in the N-terminal of pullulanases in

general. This prediction is based on the lack of further conserved regions of identity among the known pullulanases beyond the Y region as one proceeds to the N-terminus. Due to the size heterogeneity of the known pullulanases, the N-terminal regions beyond the Y region call vary between approximately 100-300 amino acids. For the B. deramificans pullulanase, a truncation of 309 residues would leave the first conserved region (Y at amino acid residue 310 in Figures 1A-1E) intact.

B. deramificans pullulanase

Mature *B. deramificans* pullulanase comprises the amino acid sequence (SEQ ID NO: 2) shown in Figures 1A-1E. The following description of characteristics refers to mature *B. deramificans* pullulanase. *B. deramificans* pullulanase has an isoelectric point of between 4.1 and 4.5, is heat stable and active in a wide temperature range. The *B. deramificans* pullulanase is active at an acid pH. This pullulanase is capable of catalyzing the hydrolysis of α-1, 6-glucosidic bonds present both in amylopectin and in pullulan. It breaks down pullulan into maltrotriose and amylopectin into amylose. The polysaccharide pullulan, which is a polymer of maltotriose units connected to each other by alpha-1,6-linkages can be obtained from Aureobasidium pullulans (Pullaria pullulans) by the procedure of Ueda et al., Applied Microbiology, 11, 211-215 1963).

B. deramificans pullulanase hydrolyses amylopectin to form oligosaccharides (maltooligosaccharides). During this hydrolysis, the formation of oligosaccharides made up of about 13 glucose units (degree of polymerization of 13, this molecule is also called "chain A") is observed, followed by the formation of oligosaccharides made up of about 47 glucose units (degree of polymerization of 47, this molecule is also called "chain B').

The oligosaccharides with chains A and B are defined with reference to D. J. MANNERS ("Structural Analysis of Starch components by Debranching Enzymes" in "New Approaches to research on Cereal Carbohydrates", Amsterdam, 1985, pages 45-54) and B. E. ENEVOLDSEN ("Aspects of the fine structure of starch" in "New Approaches to research on Cereal Carbohydrates", Amsterdam, 1985, pages 55-60).

The B. deramificans pullulanase hydrolyses potato amylopectin. This hydrolysis can be carried out with an aqueous suspension of amylopectin in the presence of the pullulanase under the conditions of optimum activity of the pullulanase, that is to say at a temperature of about 60 °C and at a pH of about 4.3.

The B. deramificans pullulanase catalyses the condensation reaction of maltose to form tetraholosides (oligosaccharides having 4 glucose units).

The B. deramificans pullulanase has a half-life of about 55 hours, measured at a temperature of about 60 °C in a solution buffered at a pH of about 4.5 and in the absence of substrate.

Half-life means that the pullulanase shows a relative enzymatic activity of at least 50%, measured after an incubation of 55 hours at a temperature of about 60 °C in a solution buffered at a pH of about 4.5 and in the absence of substrate.

The B. deramificans pullulanase is heat stable at an acid pH and shows a relative enzymatic activity of at least 55%, measured after an incubation of 40 hours at a temperature of 60 °C in a solution buffered at a pH of about 4.5 and in the absence of substrate. It shows a relative enzymatic activity of at least 70%, measured after an incubation of 24 hours under these same conditions.

Relative enzymatic activity means the ratio between the enzymatic activity measured in the course of a test carried out under the given pH, temperature, substrate and duration conditions, and the maximum enzymatic activity measured in the course of this same test, the enzymatic activity being measured starting from the hydrolysis of pullulane and the maximum enzymatic activity being fixed arbitrarily at the value of 100.

The B. deramificans pullulanase is furthermore stable in a wide range of acid pH values. Under the conditions described below, it is active at a pH greater than or equal to 3. In fact, the B. deramificans pullulanase shows a relative enzymatic activity of at least 85%, measured after an incubation of 60 minutes at a temperature of about 60 °C in the absence of substrate and in a pH range greater than or equal to about 3.5.

Under the conditions described below, it is active at a pH of less than or equal to 7. In fact, the B. deramificans pullulanase shows a relative enzymatic activity of at least 85%, measured after an incubation of 60 minutes at a temperature of about 60 °C in the absence of substrate and in a pH range less than or equal to about 5.8.

It preferably shows a relative enzymatic activity of greater than 90%, measured in a pH range of between about 3.8 and about 5 under these same conditions.

The B. deramificans pullulanase develops an optimum enzymatic activity, measured at a temperature of about 60 °C, in a pH range greater than 4.0. It develops an optimum enzymatic activity, measured at a temperature of about 60 °C, in a pH range less than 4.8. The B. deramificans pullulanase preferably develops an optimum enzymatic activity, measured at a temperature of about 60 °C, at a pH of about 4.3. Furthermore, it develops an optimum enzymatic activity, measured at a pH of about 4.3, in a temperature range of between 55 and 65 °C, and more particularly at 60 °C.

The B. deramificans pullulanase develops an enzymatic activity of more than 80% of the maximum enzymatic activity (the maximum enzymatic activity being measured at a temperature of 60 °C and at a pH of 4.3) in a pH range between about 3.8 and about 4.9 at a temperature of about 60 °C.

The strain *Bacillus deramificans* T 89.117D has been deposited in the collection called BELGIAN CORRDINATED COLLECTIONS OF MICROORGANISM (LMG culture collection, University of Ghent, Laboratory of Microbiology - K. L. Ledeganckstraat 35, B - 9000 GHENT, Belgium) in accordance with the Treaty of Budapest under number LMG P-13056 on 21 June 1993.

II. Expression Systems

The present invention provides host cells, expression methods and systems for the production and secretion of modified pullulanase in gram-positive microorganisms and gram-negative microorganisms. In one embodiment, a host cell is genetically engineered to comprise nucleic acid encoding a modified pullulanase. In another embodiment, the host cell is genetically engineered to comprise nucleic acid encoding a full length or mature pullulanase, which upon culturing produces a modified pullulanase. In a preferred embodiment, the host cell is a member of the genus Bacillus which has been modified to have a mutation or deletion of endogenous proteases.

Inactivation of a protease in a host cell

Producing an expression host cell incapable of producing a naturally occurring protease necessitates the replacement and/or inactivation of the naturally occurring gene from the genome of the host cell. In a preferred embodiment, the mutation is a non-reverting mutation.

One method for mutating nucleic acid encoding a protease is to clone the nucleic acid or part thereof, modify the nucleic acid by site directed mutagenesis and reintroduce the mutated nucleic acid into the cell on a plasmid. By homologous recombination, the mutated gene may be introduced into the chromosome. In the parent host cell, the result is that the naturally occurring nucleic acid and the mutated nucleic acid are located in tandem on the chromosome. After a second recombination, the modified sequence is left in the chromosome having thereby effectively introduced the mutation into the chromosomal gene for progeny of the parent host cell.

Another method for inactivating the protease proteolytic activity is through deleting the chromosomal gene copy. In a preferred embodiment, the entire gene is deleted, the deletion occurring in such as way as to make reversion impossible. In another preferred embodiment, a partial deletion is produced, provided that the nucleic acid sequence left in the chromosome is too short for homologous recombination with a plasmid encoded metallo-protease gene. In another preferred embodiment, nucleic acid encoding the catalytic amino acid residues are deleted.

Deletion of the naturally occurring microorganism protease can be carried out as follows. A protease gene including its 5' and 3' regions is isolated and inserted into a cloning vector. The coding region of the protease gene is deleted from the vector *in vitro*, leaving behind a sufficient amount of the 5' and 3' flanking sequences to provide for homologous recombination with the naturally occurring gene in the parent host cell. The vector is then transformed into the host cell. The vector integrates into the chromosome via homologous recombination in the flanking regions. This method leads to a strain in which the protease gene has been deleted.

The vector used in an integration method is preferably a plasmid. A selectable marker may be included to allow for ease of identification of desired recombinant microorgansims. Additionally, as will be appreciated by one of skill in the art, the vector is preferably one which can be selectively integrated into the chromosome. This can be achieved by introducing an inducible origin of replication, for example, a temperature sensitive origin into the plasmid. By growing the transformants at a temperature to which the origin of replication is sensitive, the replication function of the plasmid is inactivated, thereby providing a means for

selection of chromosomal integrants. Integrants may be selected for growth at high temperatures in the presence of the selectable marker, such as an antibiotic. Integration mechanisms are described in WO 88/06623.

Integration by the Campbell-type mechanism can take place in the 5' flanking region of the protease gene, resulting in a protease positive strain carrying the entire plasmid vector in the chromosome in the pullulanase locus. Since illegitimate recombination will give different results it will be necessary to determine whether the complete gene has been deleted, such as through nucleic acid sequencing or restriction maps.

Another method of inactivating the naturally occurring protease gene is to mutagenize the chromosomal gene copy by transforming a microorganism with oligonucleotides which are mutagenic. Alternatively, the chromosomal protease gene can be replaced with a mutant gene by homologous recombination.

The present invention encompasses Bacillus host cells having protease deletions or mutations, such as deletions or mutations in apr, npr, epr, mpr, isp and/or bpf and/or others known to those of skill in the art. Disclosure relating to deleting protease(s) in the gram-positive microorganism, Bacillus, can be found in United States Patent Application Nos. 5,264,366; 5,585,253; 5,620,880 and European Patent No. EP 0369 817 B1

One assay for the detection of mutants involves growing the *Bacillus* host cell on medium containing a protease substrate and measuring the appearance or lack thereof, of a zone of clearing or halo around the colonies. Host cells which have an inactive protease will exhibit little or no halo around the colonies.

III. <u>Production of modified pullulanase</u>

For production of modified pullulanase in a host cell, an expression vector comprising at least one copy of nucleic acid encoding a modified pullulanase, and preferably comprising multiple copies, is transformed into the host cell under conditions suitable for expression of the modified pullulanase. In accordance with the present invention, polynucleotides which encode a modified pullulanase, or fusion proteins or polynucleotide homolog sequences that encode amino acid variants of modified pullulanase (as long as the variant retains the ability to catalyse the hydrolysis of a α-1, 6 - glucosidic bond), may be used to generate recombinant DNA molecules that direct their expression in host cells. A host cell may be a gram-positive or a gram-negative cell. In one embodiment, the host cell belongs to the genus *Bacillus*. In another embodiment, the Bacillus host cell includes *B.subtilis*, *B.*

licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus and Bacillus thuringiensis. In a preferred embodiment, the gram positive host cell is *Bacillus licheniformis.*

As will be understood by those of skill in the art, it may be advantageous to produce polynucleotide sequences possessing non-naturally occurring codons. Codons preferred by a particular gram-positive host cell (Murray E et al (1989) Nuc Acids Res 17:477-508) can be selected, for example, to increase the rate of expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

Altered pullulanase polynucleotide sequences which may be used in accordance with the invention include deletions, insertions or substitutions of different nucleotide residues resulting in a polynucleotide that encodes the same or a functionally equivalent modified pullulanase. As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

As used herein an "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring modified pullulanase.

As used herein "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

The encoded protein may also show deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent modified pullulanase. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the variant retains the ability to modulate secretion. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine, phenylalanine, and tyrosine.

The polynucleotides encoding a modified pullulanase of the present invention may be engineered in order to modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, eg, site-directed mutagenesis to insert

new restriction sites, to alter glycosylation patterns or to change codon preference, for example.

In one embodiment of the present invention, a polynucleotide encoding a modified pullulanase may be ligated to a heterologous sequence to encode a fusion protein. A fusion protein may also be engineered to contain a cleavage site located between the modified pullulanase nucleotide sequence and the heterologous protein sequence, so that the modified pullulanase may be cleaved and purified away from the heterologous fusion partner.

IV. Vector Sequences

Expression vectors used in expressing the pullulanases of the present invention in host microorganisms comprise at least one promoter associated with a modified pullulanase, which promoter is functional in the host cell. In one embodiment of the present invention, the promoter is the wild-type promoter for the selected pullulanase and in another embodiment of the present invention, the promoter is heterologous to the pullulanase, but still functional in the host cell. In one embodiment of the present invention, nucleic acid encoding the modified pullulanase is stably integrated into the microorganism genome.

In a preferred embodiment, the expression vector contains a multiple cloning site cassette which preferably comprises at least one restriction endonuclease site unique to the vector, to facilitate ease of nucleic acid manipulation. In a preferred embodiment, the vector also comprises one or more selectable markers. As used herein, the term selectable marker refers to a gene capable of expression in the host microorganism which allows for ease of selection of those hosts containing the vector. Examples of such selectable markers include but are not limited to antibiotics, such as, erythromycin, actinomycin, chloramphenicol and tetracycline.

V. Transformation

A variety of host cells can be used for the production of modified pullulanase including bacterial, fungal, mammalian and insects cells. General transformation procedures are taught in Current Protocols In Molecular Biology (vol. 1, edited by Ausubel et al., John Wiley & Sons, Inc. 1987, Chapter 9) and include calcium phosphate methods, transformation using DEAE-Dextran and electroporation. Plant transformation methods are taught in Rodriquez (WO 95/14099, published 26 May 1995).

In a preferred embodiment, the host cell is a gram-positive microorganism and in another preferred embodiment, the host cell is *Bacillus*. In a further preferred embodiment the Bacillus host is *Bacillus licheniformis*. In one embodiment of the present invention, nucleic acid encoding a modified pullulanase of the present invention is introduced into a host cell via an expression vector capable of replicating within the *Bacillus* host cell. Suitable replicating plasmids for *Bacillus* are described in Molecular Biological Methods for *Bacillus*, Ed. Harwood and Cutting, John Wiley & Sons, 1990; see chapter 3 on plasmids.

Suitable replicating plasmids for *B. subtilis* are listed on page 92.

In another embodiment, nucleic acid encoding a modified pullulanase of the present invention is stably integrated into the host microorganism genome. Preferred host cells are gram-positive host cells. Another preferred host is *Bacillus*. Bacillus host cells include *B.subtilis*, *B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus and Bacillus thuringiensis.* A preferred host is *Bacillus subtilis*. Another preferred host is *B. licheniformis*. Several strategies have been described in the literature for the direct cloning of DNA in *Bacillus*. Plasmid marker rescue transformation involves the uptake of a donor plasmid by competent cells carrying a partially homologous resident plasmid (Contente et al., Plasmid 2:555-571 (1979); Haima et al., Mol. Gen. Genet. 223:185-191 (1990); Weinrauch et al., J. Bacteriol. 154(3):1077-1087 (1983); and Weinrauch et al., J. Bacteriol. 169(3):1205-1211 (1987)). The incoming donor plasmid recombines with the homologous region of the resident "helper" plasmid in a process that mimics chromosomal transformation.

Transformation by protoplast transformation is described for *B. subtilis* in Chang and Cohen, (1979) Mol. Gen. Genet 168:111-115; for B.megaterium in Vorobjeva et al., (1980) FEMS Microbiol. Letters 7:261-263; for B. amyloliquefaciens in Smith et al., (1986) Appl. and Env. Microbiol. 51:634; for B.thuringiensis in Fisher et al., (1981) Arch. Microbiol. 139:213-217; for B.sphaericus in McDonald (1984) J. Gen. Microbiol. 130:203; and B.larvae in Bakhiet et al., (1985, Appl. Environ. Microbiol. 49:577). Mann et al., (1986, Current Microbiol. 13:131-135) report on transformation of *Bacillus* protoplasts and Holubova, (1985) Folia Microbiol. 30:97) disclose methods for introducing DNA into protoplasts using DNA containing liposomes.

VI. Identification of Transformants

Whether a host cell has been transformed with a modified or a naturally occurring gene encoding a pullulanase activity, detection of the presence/absence of marker gene expression can suggest whether the gene of interest is present However, its expression should be confirmed. For example, if the nucleic acid encoding a modified pullulanase is inserted within a marker gene sequence, recombinant cells containing the insert can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with nucleic acid encoding the pullulanase under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the pullulanase as well.

Alternatively, host cells which contain the coding sequence for a modified pullulanase and express the protein may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

The presence of the pullulanase polynucleotide sequence in a host microorganism can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes, portions or fragments of the pullulanase polynucleotide sequences.

VII. Assay of Pullulanase Activity

There are various assays known to those of skill in the art for detecting and measuring pullulanase activity. An enzymatic unit of B. deramificans pullulanase (PUN) is defined as the amount of enzyme which, at a pH of 4.5, at a temperature of 60 degrees C and in the presence of pullulane, catalyses the release of reducing sugars at a rate of 1 μM glucose equivalent per minute.

Pullulanase activity can be measured in the presence or the absence of substrate. In one aspect, pullulanase activity can be measured in the presence of substrate according to the following protocol. 1 ml of a 1% strength solution of pullulane in a 50 nM acetate buffer at pH 4.5 is incubated at 60 °C for 10 minutes. 0.1 ml of a solution of pullulanase corresponding to an activity of between 0.2 and 1 PUN/ml is added thereto. The reaction is stopped after 15 minutes by addition of 0.4 ml of 0.5 M NaOH. The reducing sugars released are analyzed by the method of

SOMOGYI-NELSON [J. Biol. Chem., 153 (1944) pages 375-380; and J. Biol. Chem., 160 (1945), pages 61-68].

Another method can be used to analyze the pullulanase. The enzymatic reaction in the presence of pullulane is carried out in accordance with the above test conditions, and is then stopped by addition of sulphuric acid (0.1 N). The hydrolysis products of pullulane are then subjected to HPLC chromatography (HPX-87H column from BIO-RAD; the mobile phase is 10 mM H_2SO_4) in order to separate the various constituents. The amount of Maltotriose formed is estimated by measurement of the area of the peak obtained.

The so-called debranching activity, that is to say the hydrolysis of the α-1, 6-glucosidic bonds present in amylopectin, can be quantified by the increase in the blue coloration caused, in the presence of iodine, by the release of amylose from amylopectin. The debranching enzymatic activity is measured in accordance with the following protocol. 0.4 ml of a 1% strength amylopectin solution containing a 50 mM acetate buffer at pH 4.5 is incubated at 60 °C for 10 minutes. The reaction is initiated by addition of 0.2 ml of pullulanase, and is stopped after 30 minutes by addition of 0.4 ml of 0.3 M HC1. 0.8 ml of a 0.0025% (v/v) strength solution of iodine is then added to 0.2 ml of this reaction mixture and the optical density is measured at 565 nm.

A preferred method is disclosed in Example IV and relies on a colorimetric method that utilizes a soluble red-pullulan substrate for the determination of pullulanase activity. As the pullulanase enzyme hydrolyzes the substrate, soluble fragments of the dyed substrate are released into the reaction solution. The substrate is then precipitated with an ethanol solution and the supernatant is evaluated for color intensity with spectrophotometer. In this assay, the degree of color intensity is proportional to the enzyme activity.

VIII. Secretion of Recombinant Proteins

Means for determining the levels of secretion of a modified pullulanase in a host microorganism and detecting secreted proteins include, using either polyclonal or monoclonal antibodies specific for the protein. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). These and other assays are described, among other places, in

Hampton R et al (1990, Serological Methods, a Laboratory Manual, APS Press, St Paul MN) and Maddox DE et al (1983, J Exp Med 158:1211).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting specific polynucleotide sequences include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the nucleotide sequence, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides.

A number of companies such as Pharmacia Biotech (Piscataway NJ), Promega (Madison WI), and US Biochemical Corp (Cleveland OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241. Also, recombinant immunoglobulins may be produced as shown in US Patent No. 4,816,567.

IX. Purification of Proteins

Host cells transformed with polynucleotide sequences encoding modified pullulanase may be cultured under conditions suitable for the expression and recovery of the pullulanase from cell culture. The protein produced by a recombinant gram-positive host cell comprising a mutation or deletion of endogenous protease activity will be secreted into the culture media. Other recombinant constructions may join the modified pullulanase polynucleotide sequences to a nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll DJ et al (1993) DNA Cell Biol 12:441-53).

Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath J (1992) Protein Expr Purif 3:263-281), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequence such as Factor XA or

enterokinase (Invitrogen, San Diego CA) between the purification domain and the heterologous protein can be used to facilitate purification.

X. Uses of The Present Invention

Modified pullulanase

A modified pullulanase of the present invention finds use in various industries including the food industry, the pharmaceuticals industry and the chemical industry. A modified pullulanase can be used in baking as an "anti-staling" agent, that is to say as an additive to prevent bread becoming stale during storage, or in brewing during production of low-calorie beers. The pullulanase can also be used in the preparation of low-calorie foods in which amylose is used as a substitute for fats. The pullulanase can be used, for example, to clarify fruit juices.

For food applications, the pullulanase can be immobilized on a support. The techniques for immobilization of enzymes are well known to the expert.

The pullulanase can also be used to hydrolyse amylopectin and to form oligosaccharides starting from this amylopectin. The pullulanase can also be used to form tetraholosides starting from maltose.

The pullulanase can also be used to condense mono- or oligo-saccharides, creating bonds of the alpha-1, 6 type. The pullulanase can be used for liquefaction of starch.

A modified pullulanase can be used in the same manner as its respective unmodified form. A modified pullulanase, which in unmodified form has activity under alkaline conditions, will retain activity under alkaline conditions. A modified pullulanase which in unmodified form has activity under acidic conditions, will retain activity under acidic conditions. A particular modified pullulanase will be formulated according to the intended uses. Stabilizers or preservatives can also be added to the enzymatic compositions comprising a modified pullulanase. For example, a modified pullulanase can be stabilized by addition of propylene glycol, ethylene glycol, glycerol, starch, pullulane, a sugar, such as glucose or sorbitol, a salt, such as sodium chloride, calcium chloride, potassium sorbate, and sodium benzoate, or a mixture of two or more of these products. The enzymatic compositions according to the invention can also comprise one or more other enzymes. Such enzymes include but are not limited to glucoamylase, alpha-amylase, beta-amylase, alpha-

glucosidase, isoamylase, cyclomaltodextrin, glucotransferase, beta-glucanase, glucose isomerase, saccharifying enzymes, and enzymes which cleave glucosidic bonds or a mixture of two or more of these. In a preferred embodiment, the enzymatic composition comprises a modified pullulanase of the present invention at 80% and a glucoamylase at 20%.

The manner and method of carrying out the present invention may be more fully understood by those of skill in the art by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

EXAMPLES

Example I:

Example I illustrates the production of a modified pullulanase as described herein. The nucleic acid sequence encoding a pullulanase is modified by recombinant DNA techniques such as standard PCR primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. (Saiki, R. K., et al., 1988, Science 239:487-491.) and PCR fusion techniques (Fleming, A. B., et al. *Appl. Environ. Microbiol.* **61**, 3775-3780). DNA encoding the desired modified pullulanase is fused to the C-terminus of a signal sequence, preferably a host microorganism signal sequence. This construct is cloned and transformed into a host cell, such as, B. subtilis or B.licheniformis, and cultured under standard fermentation conditions. The modified pullulanase is purified from the fermentation broth and assayed for activity.

Example II

Example II describes the modified forms of pullulanase obtained upon culturing the recombinant B. licheniformis host cell comprising nucleic acid encoding a mature B.deramificans pullulanase wherein the host cell has a deletion of the Carlsburg and endo GluC proteases. The B. licheniformis was cultured under standard fermentation conditions in a complex media. The fermentation broth was subjected to standard HPCL analysis and the results are shown in Figures 3A-3C which illustrate a timecourse of the various species of modified pullulanase formed during the fermentation process. Peak 1 designates the mature B. deramificans pullulanase having a molecular weight of 105 kD; peak 2 designates the modified pullulanase which has deletion of 102 amino acids from the amino terminus of mature B. deramificans pullulanase; and peak 3 designates the modified pullulanase

which has a deletion of 98 amino acids from the amino terminus as measured by standard HPLC analysis. The modified pullulanase species which has an additional amino acid on the mature sequence is not detectable by HPLC analysis but was detected upon nucleic acid sequencing. Figures 3A-3C illustrate that over fermentation time, Peak 1 corresponding to the mature B. deramificans pullulanase decreases while Peaks 2 and 3 increase. Figures 4A-4D illustrate the stability of the modified pullulanase produced upon fermentation of B.licheniformis having a deletion of the Carlsburg and endoGluC proteases. B. licheniformis comprising nucleic acid encoding a mature B. deramificans was cultured under conditions suitable for the expression and secretion of the modified pullulanase and the fermentation broth was adjusted to a pH of 4.5, 5.5 and 6.5 at room temperature. The modified pullulanase was most stable at a pH of 4.5.

Example III

Example III describes the saccharification process comparing enzymatic compositions comprising different percentages of pullulanase. Enzymatic compositions comprising either 20% glucoamylase:80%modified pullulanase (20:80) activity or 75% glucoamylase:25% pullulanase activity (75:25) were tested in saccharification processes at a concentration of 0.550, 0.635 and 0.718 liters of enzymatic composition per metric ton of dissolved solids. As shown in Figures 5A-5C, an enzymatic composition comprising 20% glucoamylase and 80% pullulanase activity is able to increase the final glucose yield without an increase in undesirable disaccharide formation. Furthermore, the absolute concentration of the 20:80 enzyme composition can be increased without the undesirable increase in disaccharide formation that is seen with the 75:25 enzyme composition or glucoamylase alone.

Example IV

Example IV describes an assay for the determination of activity of a modified pullulanase of the present invention. This assay is based on a colorimetric method that utilizes a soluble red-pullulan substrate for the determination of pullulanase activity.

Reagent Preparation

A 200mM Sodium Acetate buffer pH 5.0 w/Acarbose (density~1.010) was prepared by weighing out 16.406g of anhydrous Sodium acetate or 27.21g of Sodium acetate trihydrate and dissolving it in 900 mls of deionized water (DI) in 1 L

graduated cylinder by stirring with a magnetic stir bar. The pH was adjusted to 5.0 with glacial acetic acid. 0.300g of Acarbose was added to the solution and allowed to dissolve. The volume was brought up to 1000mL with DI water and mixed.

2% Red Pullulan Substrate Preparation

1.00g of Red Pullulan substrate was weighed out and dissolved in 50 mL of sodium acetate buffer by stirring with a magnetic stir bar for approximately 20-30 minutes. This solution is stable for two weeks stored at 4°C.

Preparation of a working standard

Using positive displacement pipettes a 1:10 dilution of the Pullulanase Standard was prepared. The assigned activity of the standard was 195.9 ASPU/ml.
The following working concentrations were prepared from the standard from the 1:10 stock dilution.

Sample Preparation

For a control, Optimax* L-300 MA7EC191 PU B1 3-19A available from Genencor International was used. The control was diluted 1:1000 in sodium acetate buffer. All samples were diluted in sodium acetate buffer to obtain final reaction absorbances that fall on the calibration curve. The sample was brought to room temperature. A minimum of 100ul of sample was used for the initial dilution.

Assay Procedure

250 ul of each standard working concentration, control and sample was placed into two appropriately labeled microcentrifuge tubes. To each tube 250 ul of 2% substrate solution was added with a repeater pipette and a 12.5 ml Combitip set on 1. The samples were Vortexed for 3 seconds and incubated at 40°C for 20 minutes.
The samples were remove from the water bath and immediately 1.0 ml of 95% EtOH was added to the samples in the same order as above. A repeater pipette and a 12.5 or 50 ml Combitip set on 4 or 1, was used. The samples were vortexed for 3 seconds. The samples were incubated at room temperature for 5-10 minutes, then centrifuged for ten minutes in a benchtop centrifuge. The supernatant of the standards and samples were read in a spectrophotometer at 510 nm using 1.5 mL cuvettes. (The spectrophotometer was zeroed with 95% EtOH)

Calculations

*Trade-mark

Using the standard concentrations and correlating absorbances (subtracting the blank absorbance), a calibration curve is developed with a computer spreadsheet, programmable calculator, or graph paper. The curve should be linear over the range of the standard concentrations with a correlation coefficient (r) of 0.998 or greater. The precision of the assay should fall between 5-10% CV. For liquids: u/ml = (u/ml from standard curve)* (sample dilution)

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 74541-62 Seq 16-DEC-09 v3.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

SEQUENCE TABLE

<110> Miller, Brian S.
Shetty, Jayarama K.

<120> Modified Forms of Pullulanase

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<140> 09/262,126
<141> 1999-03-03

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74541-62

CLAIMS:

1. A truncated pullulanase which has the amino acid sequence of SEQ ID NO:2 (Figure 1), wherein the N-terminus of said truncated pullulanase is amino acid residue 99 or amino acid residue 103 of SEQ ID NO: 2, and wherein said truncated pullulanase is capable of catalyzing the hydrolysis of an alpha-1,6-glucosidic bond.

2. A nucleic acid comprising a polynucleotide sequence encoding the truncated pullulanase as defined in claim 1.

3. An expression vector comprising the nucleic acid as defined in claim 2.

4. A host microorganism comprising the expression vector as defined in claim 3.

5. The host microorganism of claim 4 wherein said microorganism is a *Bacillus*.

6. The host microorganism of claim 5 wherein said *Bacillus* is *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus* or *Bacillus thuringiensis*.

7. A method for the production of a truncated pullulanase in a host cell comprising the step of culturing a recombinant microorganism comprising the nucleic acid as defined in claim 2 under conditions suitable for the production of the truncated pullulanase.

8. The method of claim 7 further comprising the step of recovering the truncated pullulanase.

9. The method of claim 7 wherein the microorganism is a *Bacillilus*.

10. The method of claim 9 wherein the *Bacillus* is *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus,*

B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus or *Bacillus thuringiensis*.

11. The method of claim 10 wherein the *Bacillus* is a *B. licheniformis*.

12. An enzymatic composition comprising the truncated pullulanase as defined in claim 1, and a carrier or diluent.

13. The composition of claim 12 further comprising an enzyme selected from the group consisting of glucoamylase, alpha-amylase, beta-amylase, alpha-glucosidase, isoamylase, cyclomaltodextrin, glucotransferase, beta-glucanase, glucose isomerise, saccharifying enzymes, enzymes which cleave glucosidic bonds and any combination thereof.

14. The composition of claim 13 wherein the glucoamylase is obtained from an Aspergillus strain.

15. The composition of claim 14 wherein the Aspergillus strain is *Aspergillus niger*, *Aspergilus awamori* or *Aspergillus foetidus*.

16. The composition of claim 12 wherein said composition is in a solid form.

17. The composition of claim 12 wherein said composition is in a liquid form.

18. The composition of claim 12 comprising at least 60% truncated pullulanase.

19. The composition of claim 12 comprising at least 80% truncated pullulanase.

20. A process for the saccharification of starch, wherein said process allows for reduced concentrations of saccharification by –products, comprising the step of contacting aqueous liquefied starch with the enzyme composition as defined in any one of claims 12 to 19.

21. The process of claim 20 further comprising the step of heating said liquefied starch.

22. The process of claim 20 or 21 further comprising the step of recovering product.

23. The process of any one of claims 20 to 22 wherein said contacting is at a pH of about less than or equal to 7.0 and greater than or equal to 3.

24. The process of claim 23 wherein the pH is about 4.2.

25. The process of claim 21 wherein said heating is at a temperature range of between 55 and 65 degrees C.

26. The process of claim 25 wherein the temperature is about 60 degrees C.

27. *B. licheniformis* comprising the nucleic acid as defined in claim 2 wherein said *B. licheniformis* comprises a first gene encoding Carlsberg protease and a second gene encoding endo Glu C protease, wherein one of or both the first and second gene which codes for the protease(s) having been altered such that the protease activity is eliminated.

SMART & BIGGAR
OTTAWA, CANADA

PATENT AGENTS

GATGGGAACACGACAACGATCATTGTCCACTATTTTTGCCCTGCTGGTGATTATCAACCTTGGAGTCTAT 70

Asp Gly Asn Thr Thr Thr Ile Ile Val His Tyr Phe Cys Pro Ala Gly Asp Tyr Gln Pro Trp Ser Leu

GGATGTGGCCAAAAGACGGAGGTGGGGCTGAATACGATTTCAATCAACCGGCTGACTCTTTTGGAGCTGT 140

Trp Met Trp Pro Lys Asp Gly Gly Gly Ala Glu Tyr Asp Phe Asn Gln Pro Ala Asp Ser Phe Gly Ala Val

TGCAAGTGCTGATATTCCAGGAAACCCAAGTCAGGTAGGAATTATCGTTCGCACTCAAGATTGGACCAAA 210

Ala Ser Ala Asp Ile Pro Gly Asn Pro Ser Gln Val Gly Ile Ile Val Arg Thr Gln Asp Trp Thr Lys

GATGTGAGCGCTGACCGCTACATAGATTTAAGCAAAGGAAATGAGGTGTGGCTTGTAGAAGGAAACAGCC 280

Asp Val Ser Ala Asp Arg Tyr Ile Asp Leu Ser Lys Gly Asn Glu Val Trp Leu Val Glu Gly Asn Ser

AAATTTTTTATAATGAAAAAGATGCTGAGGATGCAGCTAAACCCGCTGTAAGCAACGCTTATTTAGATGC 350

98 102

Gln Ile Phe Tyr Asn | Glu Lys Asp Ala | Glu Asp Ala Ala Lys Pro Ala Val Ser Asn Ala Tyr Leu Asp Ala

TTCAAACCAGGTGCTGGTTAAACTTAGCCAGCCGTTAACTCTTGGGGAAGGNNNAAGCGGCTTTACGGTT 420

Ser Asn Gln Val Leu Val Lys Leu Ser Gln Pro Leu Thr Leu Gly Glu Gly ??? Ser Gly Phe Thr Val

FIG._1A

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CATGACGACACAGCAAATAAGGATATTCCAGTGACATCTGTGAAGGATGCAAGTCTTGGTCAAGATGTAA 490

His Asp Asp Thr Ala Asn Lys Asp Ile Pro Val Thr Ser Val Lys Asp Ala Ser Leu Gly Gln Asp Val

CCGCTGTTTTGGCAGGTACCTTCCAACATATTTTTGGAGGTTCCGATTGGGCACCTGATAATCACAGTAC 560

Thr Ala Val Leu Ala Gly Thr Phe Gln His Ile Phe Gly Gly Ser Asp Trp Ala Pro Asp Asn His Ser Thr

TTTATTAAAAAAGGTGACTAACAATCTCTATCAATTCTCAGGAGATCTTCCTGAAGGAAACTACCAATAT 630

Leu Leu Lys Lys Val Thr Asn Asn Leu Tyr Gln Phe Ser Gly Asp Leu Pro Glu Gly Asn Tyr Gln Tyr

AAAGTGGCTTTAAATGATAGCTGGAATAATCCGAGTTACCCATCTGACAACATTAATTTAACAGTCCCTG 700

Lys Val Ala Leu Asn Asp Ser Trp Asn Asn Pro Ser Tyr Pro Ser Asp Asn Ile Asn Leu Thr Val Pro

CCGGCGGTGCACACGTCACTTTTTCGTATATTCCGTCCACTCATGCAGTCTATGACACAATTAATAATCC 770

Ala Gly Gly Ala His Val Thr Phe Ser Tyr Ile Pro Ser Thr His Ala Val Tyr Asp Thr Ile Asn Asn Pro

TAATGCGGATTTACAAGTAGAAAGCGGGGTTAAAACGGATCTCGTGACGGTTACTCTAGGGGAAGATCCA 840

Asn Ala Asp Leu Gln Val Glu Ser Gly Val Lys Thr Asp Leu Val Thr Val Thr Leu Gly Glu Asp Pro

FIG._1B

CA 023218 2010-06-03

GATGTGAGCCATACTCTGTCCATTCAAACAGATGGCTATCAGGCAAAGCAGGTGATACCTCGTAATGTGC 910

Asp Val Ser His Thr Leu Ser Ile Gln Thr Asp Gly Tyr Gln Ala Lys Gln Val Ile Pro Arg Asn Val

TTAATTCATCACAGTACTACTATTCAGGAGATGATCTTGGGAATACCTATACACAGAAAGCAACAACCTT 980

309 Y

Leu Asn Ser Ser Gln | Tyr | Tyr Tyr | Ser Gly Asp Asp Leu Gly Asn Thr Tyr Thr Gln Lys Ala Thr | Thr Phe

TAAAGTCTGGGCACCAACTTCTACTCAAGTAAATGTTCTTCTTTATGACAGTGCAACGGGTTCTGTAACA 1050

VWAP

Lys Val Trp Ala Pro Thr | Ser Thr Gln Val Asn Val Leu Leu Tyr Asp Ser Ala Thr Gly Ser Val Thr

AAAATCGTACCTATGACGGCATCGGGCCATGGTGTGTGGGAAGCAACGGTTAATCAAAACCTTGAAAATT 1120

Lys Ile Val Pro Met Thr Ala Ser Gly His Gly Val Trp Glu Ala Thr Val Asn Gln Asn Leu Glu Asn

GGTATTACATGTATGAGGTAACAGGCCAAGGCTCTACCCGAACGGCTGTTGATCCTTATGCAACTGCGAT 1190

391 DPY

Trp Tyr Tyr Met Tyr Glu Val Thr Gly Gln Gly Ser Thr Arg Thr Ala Val | Asp Pro Tyr | Ala Thr Ala Ile

TGCACCAAATGGAACGAGAGGCATGATTGTGGACCTGGCTAAAACAGATCCTGCTGGCTGGAACAGTGAT 1260

Ala Pro Asn Gly Thr Arg Gly Met Ile Val Asp Leu Ala Lys Thr Asp Pro Ala Gly Trp Asn Ser Asp

FIG._1C

CA 02321817 2010-06-03

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AAACATATTACGCCAAAGAATATAGAAGATGAGGTCATCTATGAAATGGATGTCCGTGACTTTTCCATTG 1330

433 A

Lys His Ile Thr Pro Lys Asn Ile Glu Asp Glu Val | Ile Tyr Glu Met Asp Val Arg Asp Phe | Ser Ile

ACCCTAATTCGGGTATGAAAAATAAAGGGAAGTATTTGGCTCTTACAGAAAAAGGAACAAAGGGCCCTGA 1400

B

Asp Pro Asn Ser Gly Met Lys Asn | Lys Gly Lys Tyr Leu Ala Leu Thr | Glu Lys Gly Thr Lys Gly Pro Asp

CAACGTAAAGACGGGGATAGATTCCTTAAAACAACTTGGGATTACTCATGTTCAGCTTATGCCTGTTTTC 1470

C

Asn Val Lys Thr Gly Ile Asp Ser Leu Lys Gln Leu | Gly Ile Thr His Val Gln Leu Met Pro Val Phe

GCATCTAACAGTGTCGATGAAACTGATCCAACCCAAGATAATTGGGGTTATGACCCTCGCAACTATGATG 1540

YNWGY

Ala Ser Asn Ser Val Asp | Glu Thr Asp Pro Thr Gln | Asp Asn Trp Gly Tyr | Asp Pro Arg Asn Tyr Asp

TTCCTGAAGGGCAGTATGCTACAAATGCGAATGGTAATGCTCGTATAAAAGAGTTTAAGGAAATGGTTCT 1610

Val Pro Glu Gly Gln Tyr Ala Thr Asn Ala Asn Gly Asn Ala Arg Ile Lys Glu Phe Lys Glu Met Val Leu

TTCACTCCATCGTGAACACATTGGGGTTAACATGGATGTTGTCTATAATCATACCTTTGCCACGCAAATC 1680

547 I

Ser Leu His Arg Glu His Ile Gly | Val Asn Met Asp Val Val Tyr Asn His Thr Phe | Ala Thr Gln Ile

FIG._1D

TCTGACTTCGATAAAATTGTACCAGAATATTATTACCGTACGATGATCCAGGTAATTATACCAACGGATC 1750

D

Ser Asp Phe Asp Lys | Ile Val Pro Glu Tyr Tyr Tyr Arg Thr Met | Ile Gln Val Ile Ile Pro Thr Asp

AGGTACTGGAAATGAAATTGCANGCNGAAAGGCCAATGGTTCAAAAATTTATTATTGATTCCCTTAAGTA 1820

Gln Val Leu Glu Met Lys Leu ??? Ala Glu Arg Pro Met Val Gln Lys Phe Ile Ile Asp Ser Leu Lys Tyr

TTGGGTCAATGAGTATCATATTGACGGCTTCCGTTTTGACTTAATGGCGCTGCTTGGAAAAGACACGATG 1890

II

Trp Val Asn | Glu Tyr His Ile Asp Gly Phe Arg Phe Asp Leu Met Ala | Leu Leu Gly Lys Asp Thr Met

TCCAAAGCTGCCTCGGAGCTTCATGCTATTAATCCAGGAATTGCACTTTACGGTGAGCCATGGACGGGTG 1960

III

Ser Lys Ala Ala Ser Glu Leu His Ala Ile Asn Pro Gly Ile Ala Leu | Tyr Gly Glu Pro Trp Thr | Gly

GAACCTCTGCACTGCCAGATGATCAGCTTCTGACAAAAGGAGCTCAAAAAGGCATGGGAGTAGCGGTGTT 2030

Gly Thr Ser Ala Leu Pro Asp Asp Gln Leu Leu Thr Lys Gly Ala Gln Lys Gly Met Gly Val Ala Val Phe

TAATGACAATTTACGAAACGCGTTGGACGGCAATGTCTTTGATTCTTCCGCTCAAGGTTTTGCGACAGGT 2100

Asn Asp Asn Leu Arg Asn Ala Leu Asp Gly Asn Val Phe Asp Ser Ser Ala Gln Gly Phe Ala Thr Gly

FIG._1E

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GCAACAGGCTTAACTGATGCAATTAAGAATGGCGTTGAGGGGAGTATTAATGACTTTACCTCTTCACCAG 2170

Ala Thr Gly Leu Thr Asp Ala Ile Lys Asn Gly Val Glu Gly Ser Ile Asn Asp Phe Thr Ser Ser Pro

GTGAGACAATTAACTATGTCACAAGTCATGATAACTACACCCTTTGGGACAAAATAGCCCTAAGCAATCC 2240

IV

Gly Glu Thr | Ile Asn Tyr Val Thr Ser His Asp Asn Tyr Thr Leu Trp Asp Lys | Ile Ala Leu Ser Asn Pro

TAATGATTCCGAAGCGGATCGGATTAAAATGGATGAACTCGCACAAGCAGTTGTTATGACCTCACAAGGC 2310

E

Asn Asp Ser Glu Ala Asp Arg Ile Lys Met Asp Glu Leu Ala Gln Ala Val Val | Met Thr Ser Gln Gly

GTTCCATTCATGCAAGGCGGGGAAGAAATGCTTCGTANAAAAGGCGGCAACGACAATAGTTATAATGCAG 2380

Val Pro Phe Met Gln | Gly Gly Glu Glu Met Leu Arg ??? Lys Gly Gly Asn Asp Asn Ser Tyr Asn Ala

GCGATGCGGTCAATGAGTTTGATTGGAGCAGGAAAGCTCAATATCCAGATGTTTTCAACTATTATAGCGG 2450

Gly Asp Ala Val Asn Glu Phe Asp Trp Ser Arg Lys Ala Gln Tyr Pro Asp Val Phe Asn Tyr Tyr Ser Gly

GCTAATCCACCTTCGTCTTGATCACCCAGCCTTCCGCATGACGACAGCTAATGAAATCAATAGCCACCTC 2520

Leu Ile His Leu Arg Leu Asp His Pro Ala Phe Arg Met Thr Thr Ala Asn Glu Ile Asn Ser His Leu

FIG._1F

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CAATTCCTAAATAGTCCAGAGAACACAGTGGCCTATGAATTAACTGATCATGTTAATAAAGACAAATGGG 2590

Gln Phe Leu Asn Ser Pro Glu Asn Thr Val Ala Tyr Glu Leu Thr Asp His Val Asn Lys Asp Lys Trp

GAAATATCATTGTTGTTTATAACCCAAATAAAACTGTAGCAACCATCAATTTGCCGAGCGGGAAATGGGC 2660

Gly Asn Ile Ile Val Val Tyr Asn Pro Asn Lys Thr Val Ala Thr Ile Asn Leu Pro Ser Gly Lys Trp Ala

AATCAATGCTACGAGCGGTAAGGTAGGAGAATCCACCCTTGGTCAAGCAGAGGGAAGTGTCCAAGTACCA 2730

Ile Asn Ala Thr Ser Gly Lys Val Gly Glu Ser Thr Leu Gly Gln Ala Glu Gly Ser Val Gln Val Pro

GGTATATCTATGATGATCCTTCATCAAGAGGTAAGCCCAGACCACGGTAAAAAGTAATAGAAAA 2794

Gly Ile Ser Met Met Ile Leu His Gln Glu Val Ser Pro Asp His Gly Lys Lys

FIG._1G

```
                10        20        30        40
     MXXXXXXXXXXXXLVLXXXFXXXGXSXXXXGNXXT----Y  Majority
1    MAKKLIYVCLSVCLVLTWAFNVKGQSAHADGNTTTIIVHY  pullseqsig.seq.PRO
1    MLRYTRNALVLGSLVLLSGCD-NGSSSSSSGNPDT-----  klebpnseqsig.seq. pro
1    M------------VSIRRSFEA-----------------Y  subpull.seq.pro

                50        60        70        80
     ----XDXQXXXXXX-PXXXXXXEXX--XXXXXXXXVXXXD  Majority
41   FCPAGDYQPWSLWMWPKDGGGAEYDFNQPADSFGAVASAD  pullseqsig.seq.PRO
35   ----PDNQDVVVRL-PDVAVPGEAV--TAVENQAVIHLVD  klebpnseqsig.seq. pro
12   -----------------------------------VDDMN  subpull.seq.pro

                90       100       110       120
     IXGXXSXXXXXXXXXX---WXKEXXXXXXXXXXXXNXVXX  Majority
81   IPGNPSQVGIIVRTQD---WTKDVSADRYIDLSKGNEVWL  pullseqsig.seq.PRO
68   IAGITSSSAADYSSKNLYLWNNETCDALSAPVADWNDVST  klebpnseqsig.seq. pro
17   I------ITVLIPAEQ-----KEI----------------  subpull.seq.pro

               130       140       150       160
                 98  102
     XXXXSXXXXXXXXXXXXXXXXXXNXXXXXXXXXLXXXXXP  Majority
118  VEGNSQIFYN|EKDA|EDAAKPAVSNAYLDASNQVLVKLSQP  pullseqsig.seq.PRO
108  TPSGSDKYGPYWVIPLNKESGCINVIVRDGTDKLIDS---  klebpnseqsig.seq. pro
30   ------------------------------------MTPP  subpull.seq.pro
```

FIG._2A

```
        X X L X X X X X D F T - - - - - - X X X X P V X X X X X X S L X X X X X X X X X   Majority
                          170                 180                 190                 200
158     L T L G E G X S G F T V H D D T A N K D I P V T S V K D A S L G Q D V T A V L A   pullseqsig.seq.PRO
145     - D L R V A F G D F T - - - - - - D R T V S V - I A G N S A V Y D S R A D A F R   klebpnseqsig.seq. pro
34      F R L E T E I T D F - - - - - - - - - - - P L A V R E E Y S L - - - - - - - - -   subpull.seq.pro

        X X F X X X X X X X X W X - - X X X T L L - - - - - X K X X X X L Y - - - - - -   Majority
                          210                 220                 230                 240
198     G T F Q H I F G G S D W A P D N H S T L L - - - - - K K V T N N L Y Q F S G D L   pullseqsig.seq.PRO
177     A A F G V A L A E A H W V - - D K N T L L W P G G Q D K P I V R L Y - - - - - -   klebpnseqsig.seq. pro
54      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   subpull.seq.pro

        - E X X Y X Y X X X X X X X - X X X X X X X X X X X L T V X X X X X X V T F X X   Majority
                          250                 260                 270                 280
233     P E G N Y Q Y K V A L N D S W N N P S Y P S D N I N L T V P A G G A H V T F S Y   pullseqsig.seq.PRO
209     - - - - Y S H S S K V A A D - G E G K F T D R Y L K L T P T T V S Q Q V S M R F   klebpnseqsig.seq. pro
54      - E A K Y K Y - - - - - - - - - - - - - - - - - - - - - V C V S D H P V T F G K   subpull.seq.pro

        I H X X X A X Y X X X X X P X - X X X V X S G X K T D L V X X A X X E D X X X X   Majority
                          290                 300                 310                 320
273     I P S T H A V Y D T I N N P N A D L Q V E S G V K T D L V T V T L G E D P D V S   pullseqsig.seq.PRO
244     P H - - L S S Y A A F K L P D - N A N V D E L L Q G E T V A I A A A E D G I L I   klebpnseqsig.seq. pro
72      I H C V R A - - - - - - - - - - - - - - S S G H K T D L Q I G A V - - - - - - -   subpull.seq.pro
```

FIG._2B

```
                XXXXIQTAGXXX ------VLXXXXYYYXGEXL--GAXYT  Majority
                          330       340       Y 350       360
313 HTLSIQTDGYQAKQVIPRNVLNSSQY|YY|SGDDL--GNTYT  pullseqsig.seq.PRO
281 SATQVQTAG----------VLDDA-Y|AE|AAEALSYGAQLA  klebpnseqsig.seq. pro
91  ----IRTAAFDD----------EF|YY|DGE-L--GAVYT  subpull.seq.pro

                XXXTTFKVWAPTATQVXVXLYXXKXXXXXXXXMTXSXXG  Majority
                     VWAP 370       380       390       400
351 QKAT|TFKVWAPT|STQVNVLLYDSATGSVTKIVPMTASGHG  pullseqsig.seq.PRO
310 DGGV|TFRVWAPT|AQQVDVVVYSADKKVIGSHPMTRDSASG  klebpnseqsig.seq. pro
112 ADHT|VFKVWAPA|ATSAAVKLSHPNKSG--RTFQMTRLEKG  subpull.seq.pro

                VWXXTVXXDLXGXXYXYXXTX-XXXXR-----EXVDPYAX  Majority
                          410       420       430     DPY 440
391 VWEATVNQNLENWYYMYEVTG-QGSTR-----TAV|DPY|AT  pullseqsig.seq.PRO
350 AWSWQGGSDLKGAFYRYAMTVYHPQSRKVEQYEVT|DPY|AH  klebpnseqsig.seq. pro
150 VYAVTVTGDLHGYEYLFCICN-NSEWM-----ETV|DQY|AK  subpull.seq.pro

                AXXXNGEXGXVVDLXXXD--PXGWXXXXXPXXXXXXDXV-  Majority
                          450       460       470       480
425 AIAPNGTRGMIVDLAKTD--PAGWNSDKHITPKNIEDEV-  pullseqsig.seq.PRO
390 SLSTNSEYSQVVDLNDSALKPDGWDNLTMPHAQKTKADLA  klebpnseqsig.seq. pro
184 AVTVNGEKGVVL---RPD--QMKWTAPLKPFSHPV-DAV-  subpull.seq.pro
```

FIG._2C

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```
                                      A    490          500          B    510          520
        - - - I Y E X H X R D F S I - D X N S G M X N K G K Y L A L T E X D T X X X X X   Majority
462     - - -|I Y E M D V R D F|S I - D P N S G M K N|K G K Y L A L T|E K G T K G P D N   pullseqsig.seq.PRO
430     K M T|I H E S H I R D L|S A W D Q T V P A E L|R G K Y L A L T|A G D S N M V Q H   klebpnseqsig.seq. pro
217     - - -|I Y E T H L R D F|S I - H E N S G M I N|K G K Y L A L T|E T D T Q T A N G   subpull.seq.pro

                               530     C    540          550          560
        X K T G X X X L K X L G V T H V E L L P V F D X A X V D E - - - - - - - - - - -   Majority
498     V K T G I D S L K Q L|G I T H V Q L M P V F A S N S V D|E - - - - - - - - - - -   pullseqsig.seq.PRO
470     L K T - - - - L S A S|G V T H V E L L P V F D L A T V N|E F S D K V A D I Q Q P   klebpnseqsig.seq. pro
253     S S S G L A Y V K E L|G V T H V E L L P V N D F A G V D|E - - - - - - - - - - -   subpull.seq.pro

                               570          580          590          600
        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - X   Majority
527     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - T   pullseqsig.seq.PRO
506     F S R L C E V N S A V K S S E F A G Y C D S G S T V E E V L N Q L K Q S D S Q D   klebpnseqsig.seq. pro
282     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - E   subpull.seq.pro

                               610          620          630          640
        X P - - - - - - - - - - - T D X Y N W G Y D P X H Y X V P E G S Y A T N P X G X   Majority
528     D P - - - - - - - - - - - T Q D|- N W G Y|D P R N Y D V P E G Q Y A T N A N G -   pullseqsig.seq.PRO
546     N P Q V Q A L N T L V A Q T D S|Y N W G Y|D P F H Y T V P E G S Y A T D P E G T   klebpnseqsig.seq. pro
283     K P - - - - - - - - - - - L D A|Y N W G Y|N P L H F F A P E G S Y A S N P H D P   subpull.seq.pro
                                        YNWGY
```

FIG._2D

```
                X T R I K E F K X M I X X L H Q X - G X X V I M D V V Y N H T X A X X X S D - -  Majority
                                650                 660          I      670                 680
555  N A R I K E F K E M V L S L H R E - H I G V N M D V V Y N H T F A T Q I S D - -  pullseqsig.seq.PRO
586  - T R I K E F R T M I Q A I K Q D L G M N V I M D V V Y N H T N A A G P T D R T  klebpnseqsig.seq. pro
312  Q T R K T E L K Q M I N T L H Q H - G L R V I L D V V F N H V Y K R E N S P - -  subpull.seq.pro

                - - F D K I V P X Y Y X R X X E X X X X X X X X X X X D X A X E R R M X X K F  Majority
                          D     690                 700                 710                 720
592  - - F D K I V P E Y Y Y R T M I Q V I I P T D Q V L E M K L X A E R P M V Q K F  pullseqsig.seq.PRO
625  S V L D K I V P W Y Y Q R L N E T T G S V E S A T C C S D S A P E H R M F A K L  klebpnseqsig.seq. pro
349  - - F E K T V P G Y F F R H D E C G M P S N G T G V G N D I A S E R R M A R K F  subpull.seq.pro

                I A D S L X Y W X X E Y X I D G F R F D L M G X L X K D T X L X A X E X X X A X  Majority
                                730          II     740                 750                 760
630  I I D S L K Y W V N E Y H I D G F R F D L M A L L G K D T M S K A A S E L H A I  pullseqsig.seq.PRO
665  I A D S L A V W T T D Y K I D G F R F D L M G Y H P K A Q I L S A W E R I K A L  klebpnseqsig.seq. pro
387  I A D C V V Y W L E E Y N V D G F R F D L L G I L D I D T V L Y M K E K A T K A  subpull.seq.pro

                N P G I X L F G E G W D X X T S X X X E X X X A X X X A X K G X G I G X F N D X  Majority
                          III   770                 780                 790                 800
670  N P G I A L Y G E P W T G G T S A L P D D Q L L T K G A Q K G M G V A V F N D N  pullseqsig.seq.PRO
705  N P D I Y F F G E G W D S N Q S D R F E - - I A S Q I N L K G T G I G T F S D R  klebpnseqsig.seq. pro
427  K P G I L L F G E G W D L A T P L P H E Q K A A L A N A P R M P G I G F F N D M  subpull.seq.pro
```

FIG._2E

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```
    L R D A V X G N X - F D S X A - - - - - Q G F A X G A G X L X X A X - - - - - -  Majority
                      810                 820                 830                 840
710 L R N A L D G N V - F D S S A - - - - - Q G F A T G A T G L T D A I - - - - - -  pullseqsig.seq.PRO
743 L R D S V R G G G P F D S G D A L R Q N Q G I G S G A G V L P N E L A S L S D D  klebpnseqsig.seq. pro
467 F R D A V K G N T - F H L K A - - - - - T G F A L G N G E S A Q A V - - - - - -  subpull.seq.pro

    - - - - - - - - - X X G X A G S - - - - - - - - - - - X X X K - - - - - - - - A  Majority
                      850                 860                 870                 880
738 - - - - - - - - - K N G V E G S - - - - - - - - - - - - - - - - - - - - - - - -  pullseqsig.seq.PRO
783 Q V R H L A D L T R L G M A G N L A D F V M I D K D G A A K K G S E I D Y N G A  klebpnseqsig.seq. pro
495 - - - - - - - - - M H G I A G S - - - - - - - - - - - S G W K - - - - - - - - A  subpull.seq.pro

    X X X X X X X P X E X I N Y V X S H D N X T L W D K I S X X X P Q E X D - A X R  Majority
                      890      IV         900                 910                 920
745 I N D F T S S P G E T|I N Y V T S H D N Y T L W D|K I A L S N P N D S E - A D R  pullseqsig.seq.PRO
823 P G G Y A A D P T E V|V N Y V S K H D N Q T L W D|M I S Y K A S Q E A D L A T R  klebpnseqsig.seq. pro
507 L A P I V P E P S Q S|I N Y V E S H D N H T F W D|K M S F A L P Q E N D - S R K  subpull.seq.pro

    X X M Q X L A X A X V M L X Q G V P F X Q X G X E X L R X K X G X X N S Y X S G  Majority
                      930        E        940                 950                 960
784 I K M D E L A Q A V V|M T S Q G V P F M Q|G G E E M L R X K G G N D N S Y N A G  pullseqsig.seq.PRO
863 V R M Q A V S L A T V|M L G Q G I A F D Q|Q G S E L L R S K S F T R D S Y D S G  klebpnseqsig.seq. pro
546 R S R Q R L A V A I I|L L A Q G V P F I H|S G Q E F F R T K Q G V E N S Y Q S S  subpull.seq.pro
```

FIG._2F

```
                  970                 980                 990                 1000
     D X X N X X D W S R X X X - - - - - - - - - - - - - - - - - - - - - - - - - - -  Majority
824  D A V N E F D W S R K A Q - - - - - - - - - - - - - - - - - - - - - - - - - - -  pullseqsig.seq.PRO
903  D W F N R V D Y S L Q D N N Y N V G M P R I S D D G S N Y E V I T R V K E M V A  klebpnseqsig.seq. pro
586  D S I N Q L D W D R R E T - - - - - - - - - - - - - - - - - - - - - - - - - - -  subpull.seq.pro

                  1010                1020                1030                1040
     - - - - - - X K X X X X Y Y X X L I X L R K X H P A F R L X X A X X I X X H L X  Majority
837  - - - - - - Y P D V F N Y Y S G L I H L R L D H P A F R M T T A N E I N S H L Q  pullseqsig.seq.PRO
943  T P G E A E L K Q M T A F Y Q E L T E L R K S S P L F T L G D G S A V M K R V D  klebpnseqsig.seq. pro
599  - - - - - - F K E D V H Y I R R L I S L R K A H P A F R L R S A A D I Q R H L E  subpull.seq.pro

                  1050                1060                1070                1080
     F L N X X E X - - - - - - - - T V A Y X L X D X X X X D X W - X X I I V X X N A  Majority
871  F L N S P E N - - - - - - - - T V A Y E L T D H V N K D K W - G N I I V V Y N P  pullseqsig.seq.PRO
983  F R N T G S D Q Q A G L L V M T V D D G M K A G A S L D S R L D G L V V A I N A  klebpnseqsig.seq. pro
633  C L T L K E H - - - - - - - - L I A Y R L Y D L D E V D E W - K D I I V I H H A  subpull.seq.pro

                  1090                1100                1110                1120
     X P X S X T X N L P X G X X X X X L X A X S G X X G E X T L X X - - - - - A X G  Majority
902  N K T V A T I N L P S G K - - W A I N A T S G K V G E S T L G Q - - - - - A E G  pullseqsig.seq.PRO
1023 A P E S R T L N E F A G E T L - Q L S A I Q Q T A G E N S L A N G V Q I A A D G  klebpnseqsig.seq. pro
664  S P D S V E W R L P N D I P Y R L L C D P S G F Q E D P T - - E - - - - - I K K  subpull.seq.pro

                  1130                1140                1150
     T V X V P G - - I X X X I L X Q X X A X D X G - X K S X X - -  Majority
935  S V Q V P G - - I S M M I L H Q E V S P D H G - K K - - . . K  pullseqsig.seq.PRO
1062 T V T L P A W S V A V L E L P Q G E A Q G A G L P V S S K      klebpnseqsig.seq. pro
697  T V A V N G - - I G T V I L Y - - L A S D L - - - K S F A      subpull.seq.pro
```

FIG._2G

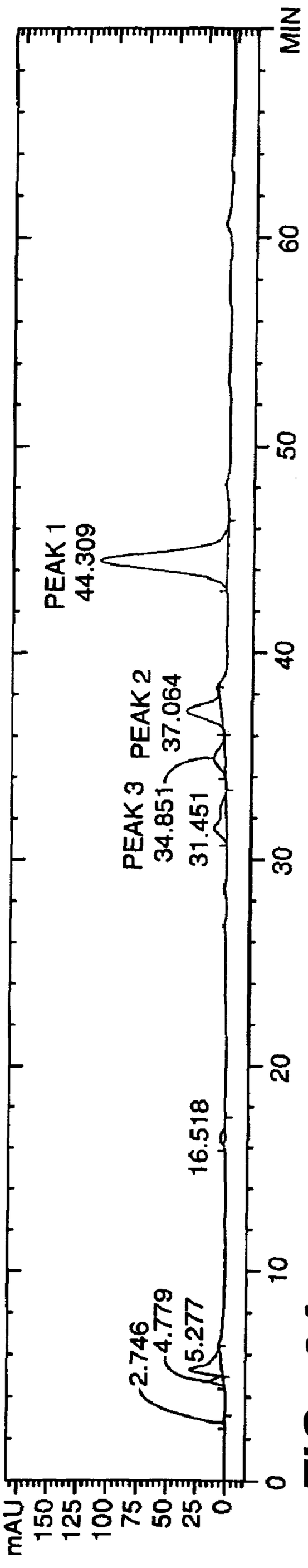
mAU
150
125
100
75
50
25
0
0
10
20
30
40
50
60
MIN
2.746
4.779
5.277
16.518
PEAK 3
34.851
PEAK 2
37.064
31.451
PEAK 1
44.309

FIG._3A

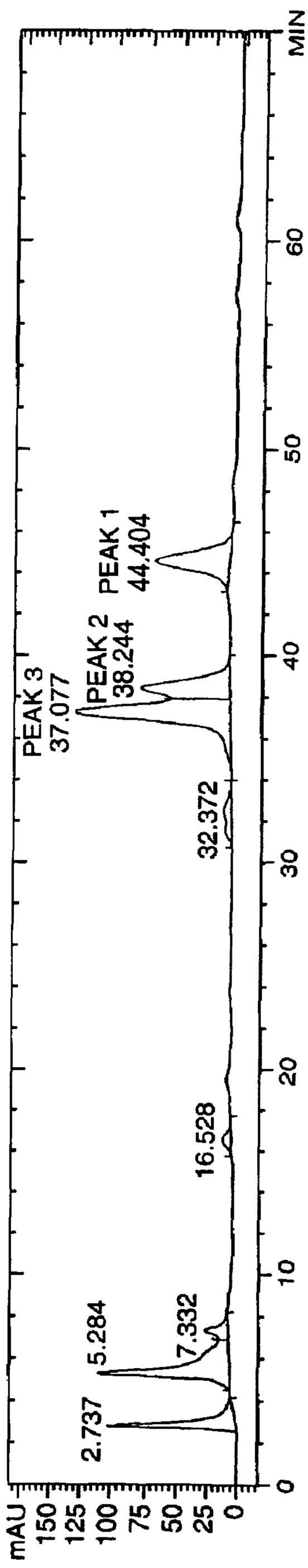
mAU
150
125
100
75
50
25
0
0
10
20
30
40
50
60
MIN
2.737
5.284
7.332
16.528
32.372
PEAK 3
37.077
PEAK 2
38.244
PEAK 1
44.404

FIG._3B

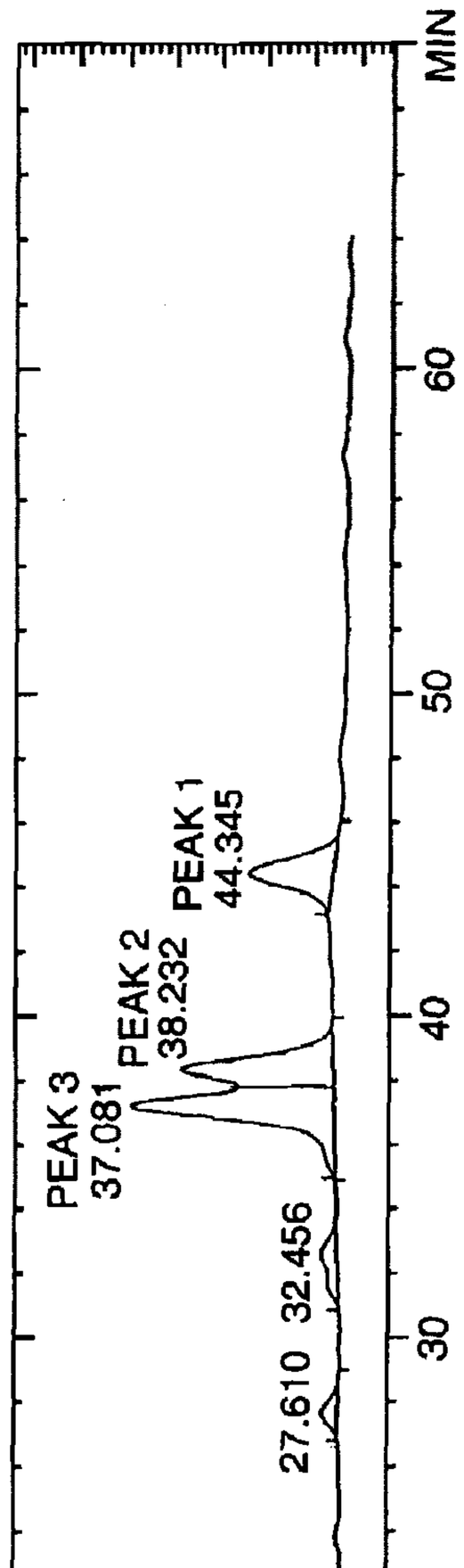
MIN
60
50
40
30
20
10
0
PEAK 1
44.345
PEAK 2
38.232
PEAK 3
37.081
32.456
27.610
16.564
11.458
10.034
8.062
5.284
2.746
mAU
150
125
100
75
50
25
0

FIG._3C

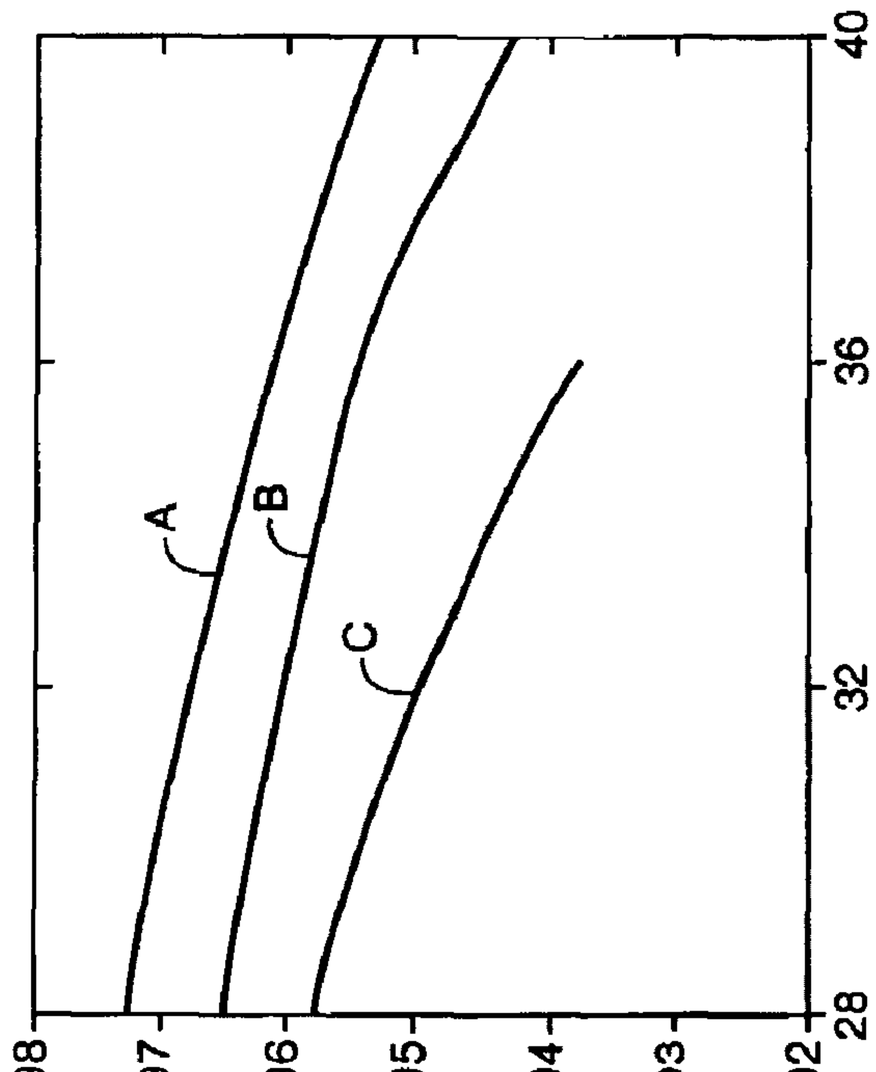
40
36
32
28
A
B
C
98
97
96
95
94
93
92
PERCENT MAXIMUM
GLUCOSE YIELD

FIG._6

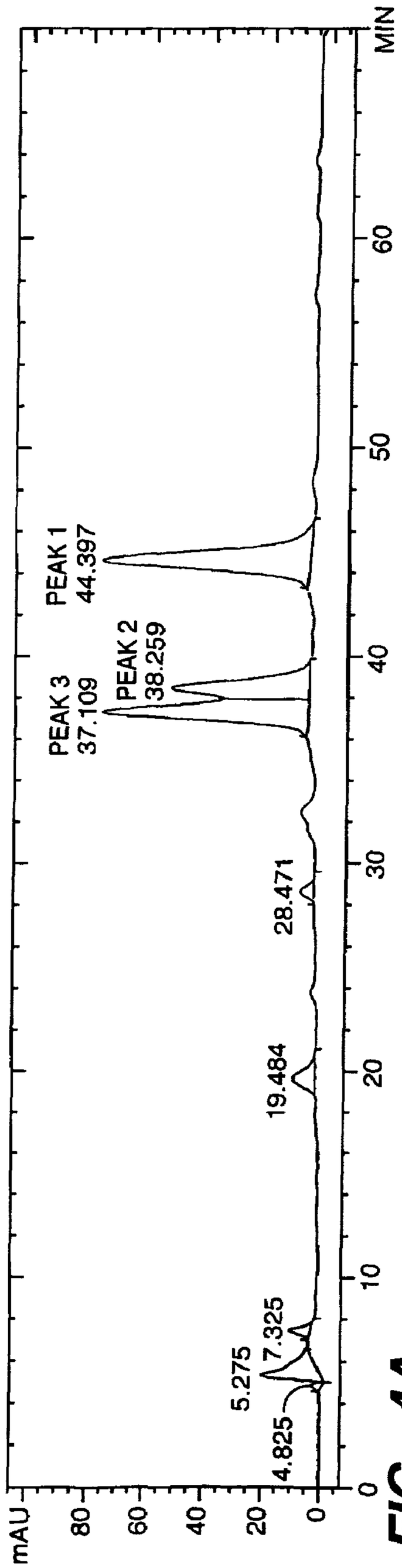
MIN
PEAK 1
44.397
PEAK 2
38.259
PEAK 3
37.109
28.471
19.484
5.275
7.325
4.825
mAU
0
20
40
60
80
10
20
30
40
50
60

FIG._4A

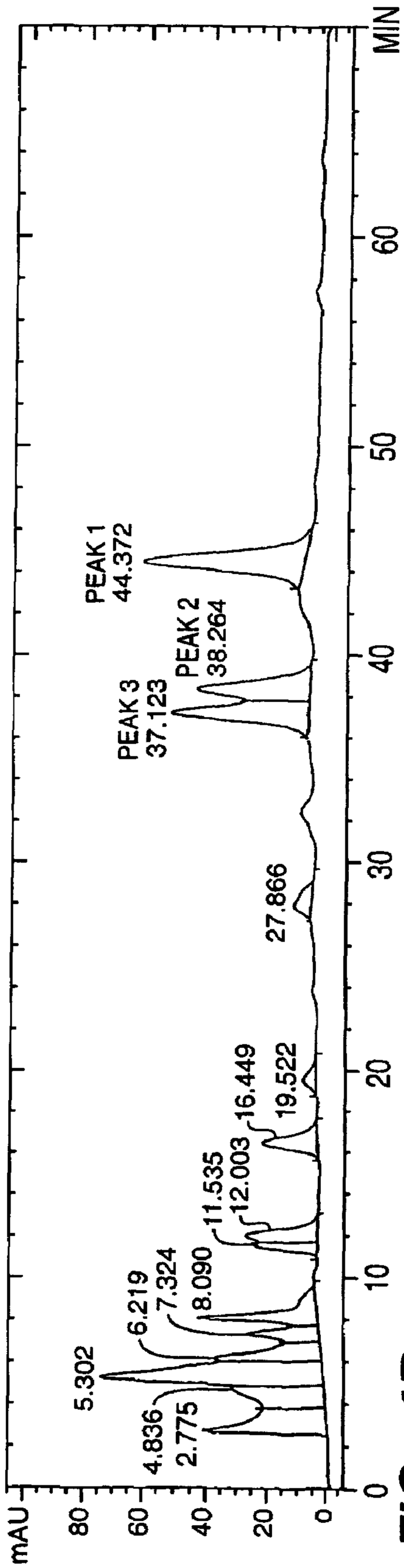
MIN
PEAK 1
44.372
PEAK 2
38.264
PEAK 3
37.123
27.866
19.522
16.449
12.003
11.535
5.302
6.219
7.324
8.090
4.836
2.775
mAU
0
20
40
60
80
10
20
30
40
50
60

FIG._4B

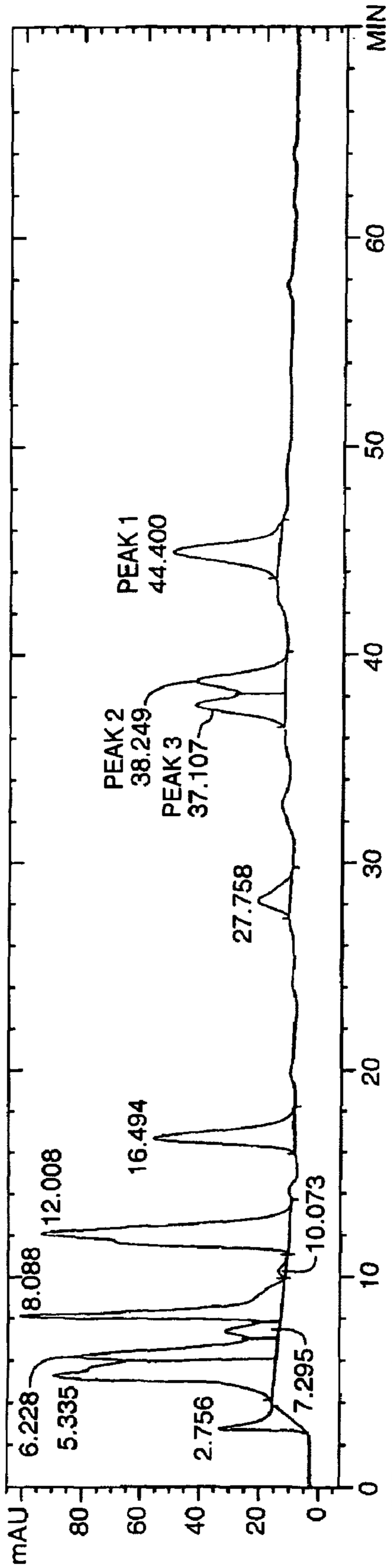
mAU
80
60
40
20
0
0
10
20
30
40
50
60
MIN
6.228
5.335
2.756
7.295
8.088
12.008
10.073
16.494
27.758
PEAK 3
37.107
PEAK 2
38.249
PEAK 1
44.400

FIG._4C

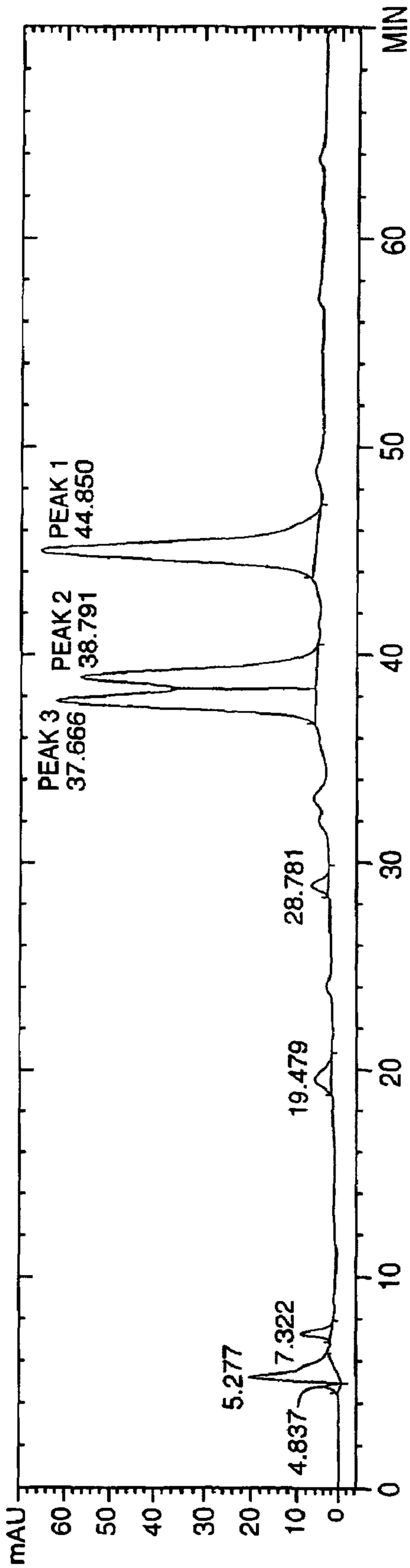
mAU
60
50
40
30
20
10
0
0
10
20
30
40
50
60
MIN
4.837
5.277
7.322
19.479
28.781
PEAK 3
37.666
PEAK 2
38.791
PEAK 1
44.850

FIG._4D

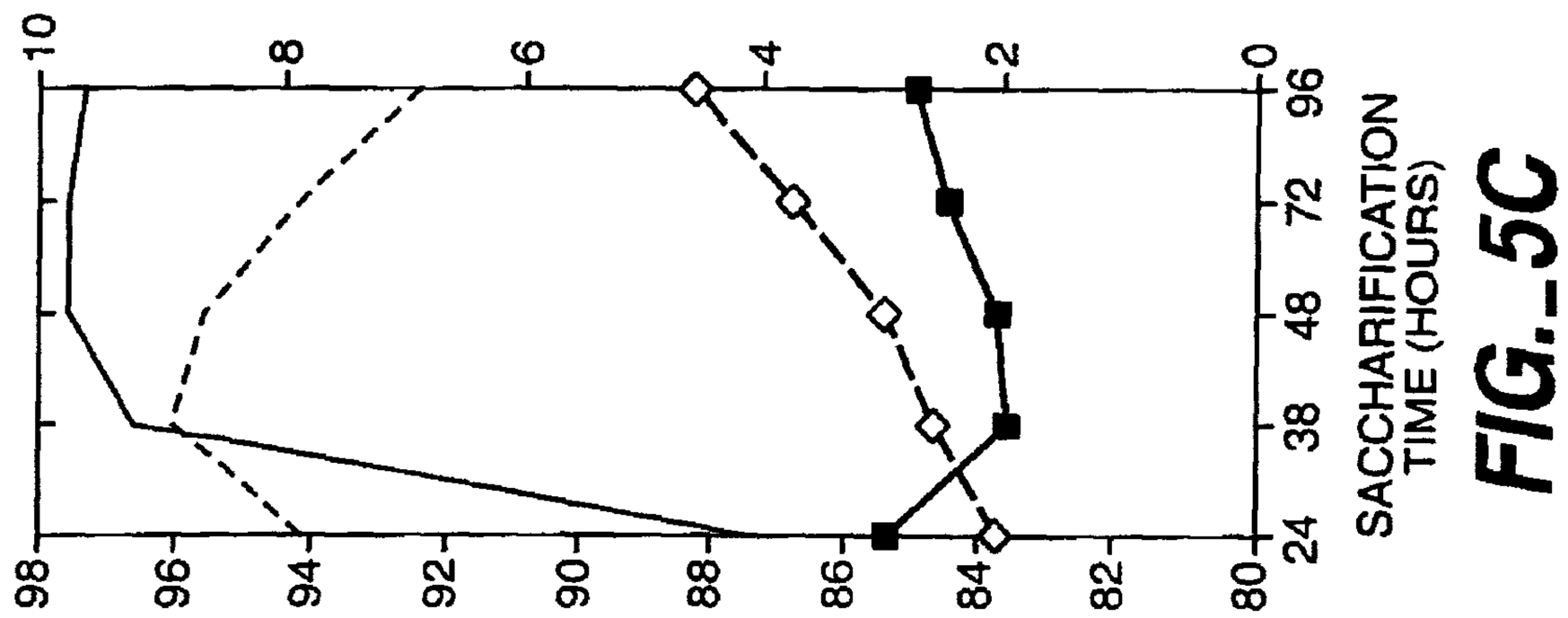

FIG._5C

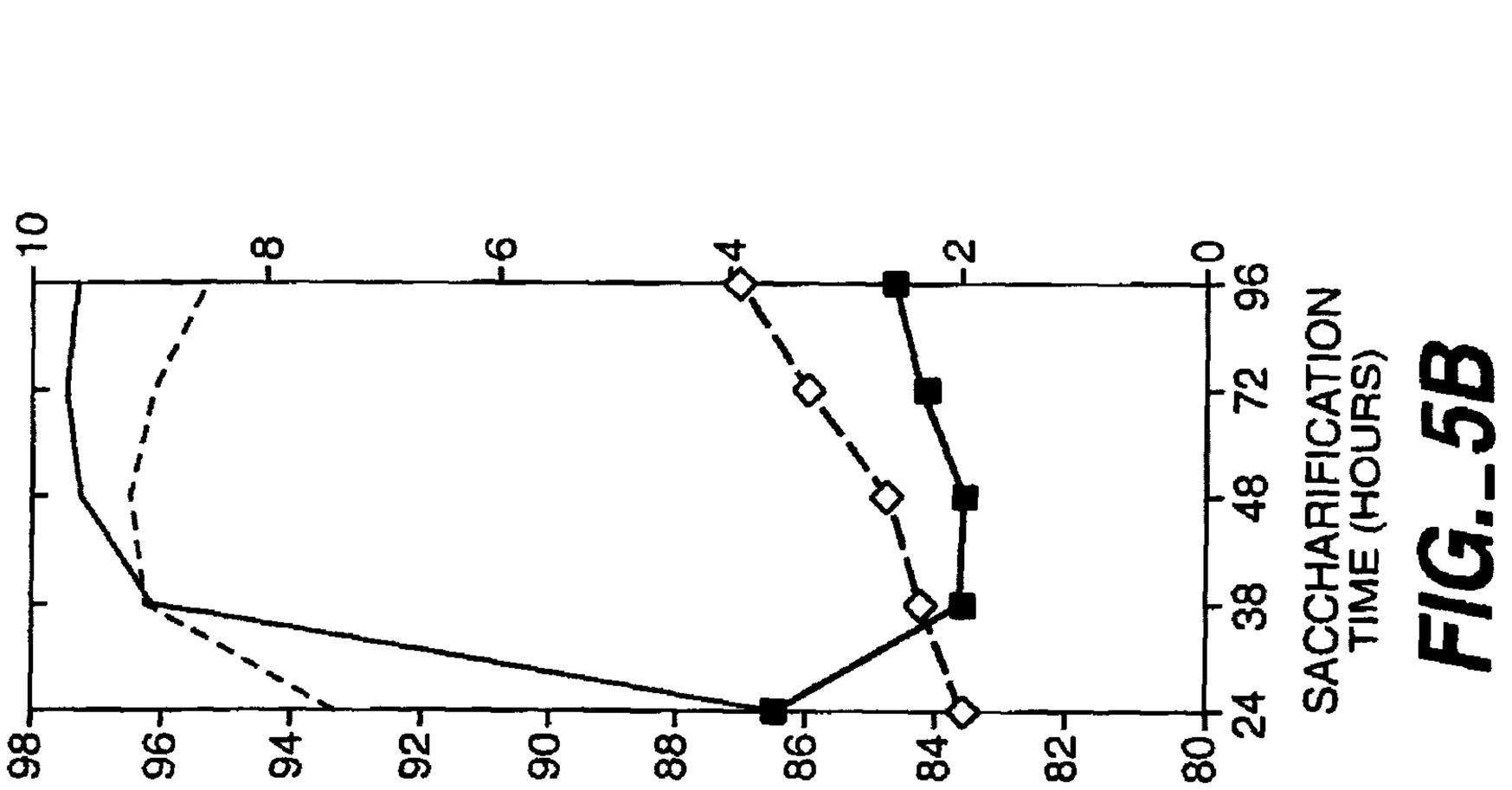

FIG._5B

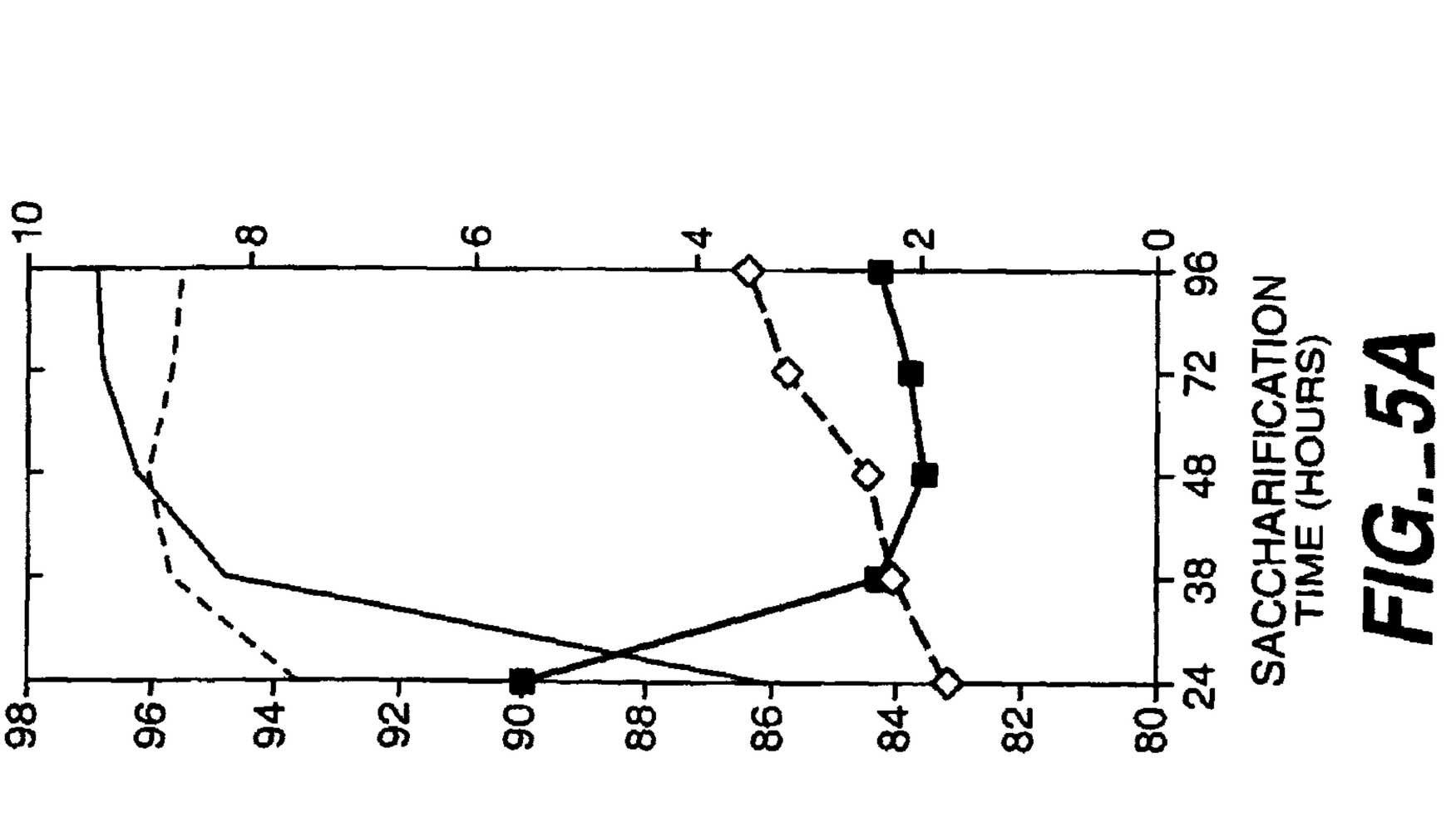

FIG._5A