

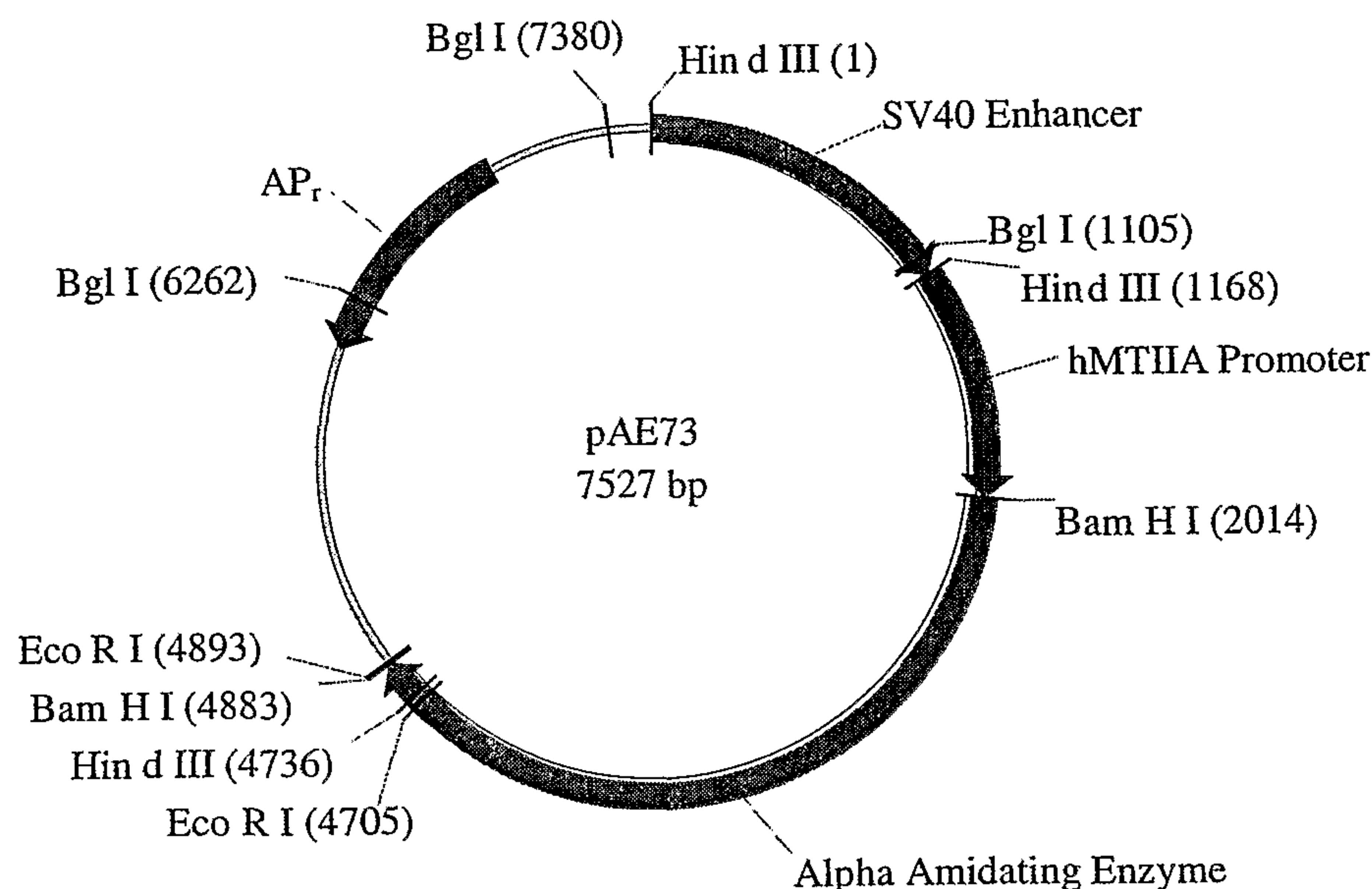


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(54) Titre : LIGNEES CELLULAIRES PERMETTANT D'EXPRIMER UNE ENZYME UTILE POUR LA PREPARATION DE PRODUITS AMIDES

(54) Title: CELL LINES FOR EXPRESSING ENZYME USEFUL IN THE PREPARATION OF AMIDATED PRODUCTS



(57) **Abrégé/Abstract:**

Cell lines are provided for expressing peptidylglycine alpha-amidating monooxygenase (PAM), or one of its two catalytic domains. High levels of enzyme expression are achieved while utilizing a non-animal source, low protein tissue culture medium. A robust two-step downstream purification results in high enzyme purity. Resulting PAM, or its PHM catalytic domain, is used to catalyze the enzymatic conversion of X-Gly to X-alpha-hydroxy-Gly or X-NH₂ (X being a peptide or any chemical compound having a carbonyl group to which a glycine group can be covalently attached). Methods of preparing preferred cell lines are also set forth.



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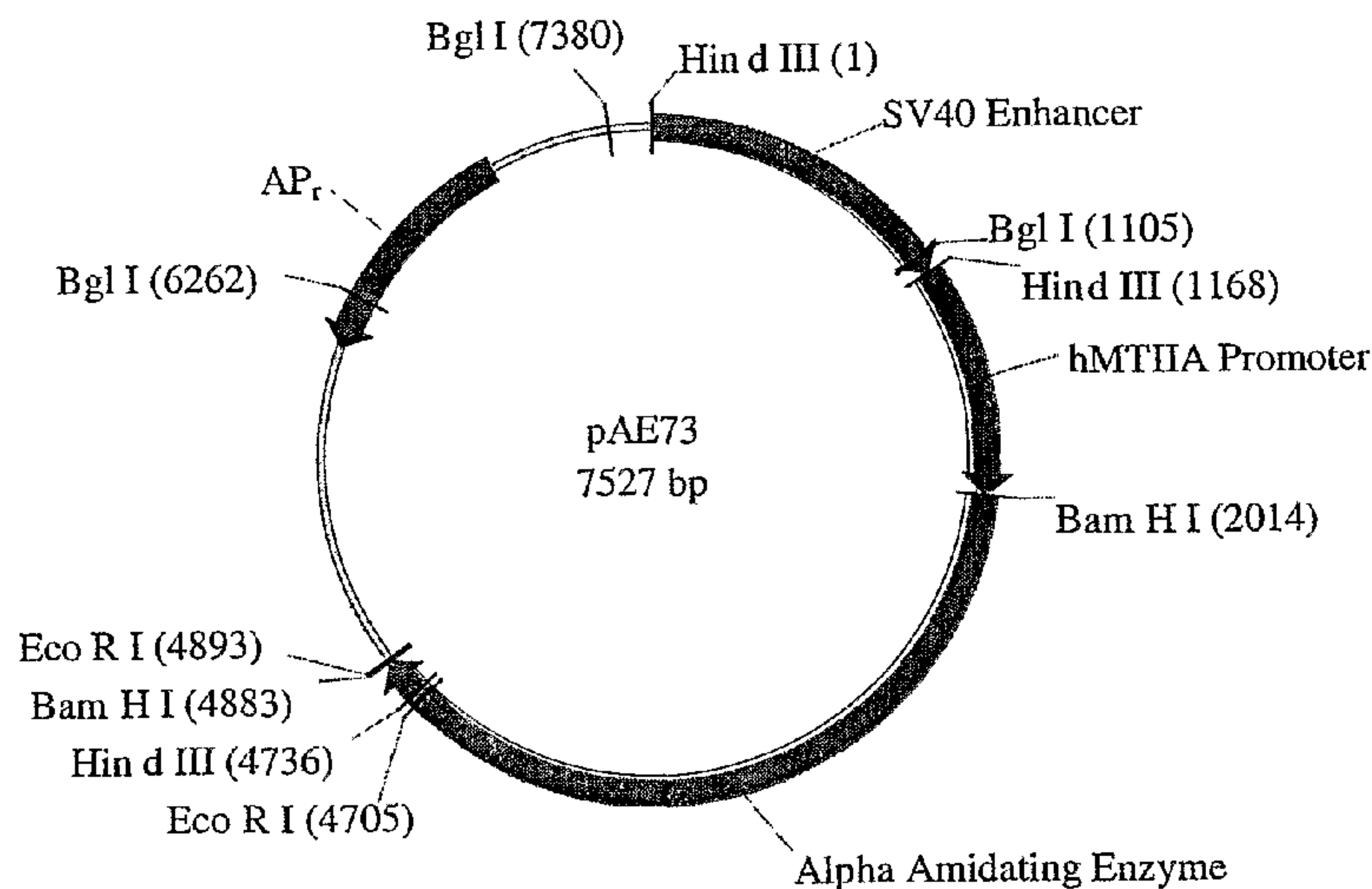
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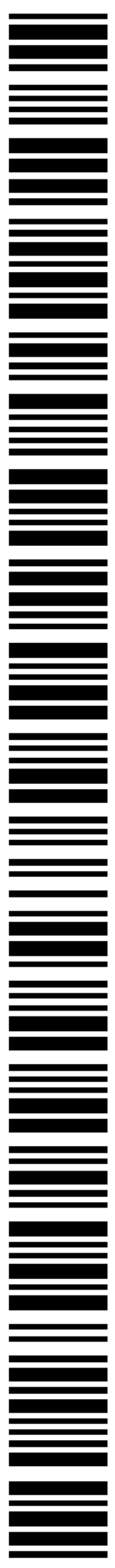
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**CELL LINES FOR EXPRESSING ENZYME USEFUL
IN THE PREPARATION OF AMIDATED PRODUCTS**

CROSS REFERENCE TO A RELATED APPLICATION

This application claims priority of U.S. Provisional Application Serial No. 60/693,612 filed June 24, 2005, the disclosure of which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to recombinant expression vectors, and to cell lines, for the expression of peptidylglycine alpha-amidating monooxygenase (PAM or α -AE), or one of its two catalytic domains. The invention also relates to the use of such PAM (or one of its catalytic domains) to catalyze the enzymatic conversion of X-Gly to X-alpha-hydroxy-Gly or X-NH₂ (X being a peptide or any chemical compound having a carbonyl group to which a glycine group can be covalently attached). The invention further relates to the preparation of preferred cell lines. In some embodiments CHO K1 hosts are utilized. In some embodiments, expression vectors include a human metallothionin IIA promoter and/or an SV40 enhancer.

DESCRIPTION OF THE RELATED ART

Numerous human hormones, growth factors, cytokines, neurotransmitters, derivatized fatty acids, and other important biological compounds have amino acid or peptide as a substantial part of their molecular structures. Many diseases respond positively to raising the level of these biological compounds in patients. Therapeutically effective amounts of such biologically relevant compounds may be administered to patients in a variety of ways. Thus, efficient cost-effective manufacturing processes for such compounds are very important. This is especially true when the biological compounds are prepared in

dosage form for oral delivery, a usually preferred mode of administration despite lower bioavailability relative to other modes of administration.

Mammalian cells and other eukaryotes can perform certain post-translational processing procedures, while prokaryotes cannot. Certain prokaryotes, such as *E. coli*, are widely employed as hosts for the production of mammalian proteins via recombinant DNA (rDNA) technology because they can be readily grown in batch fermentation procedures and because they are genetically well-characterized. However, many mammalian proteins require some type of post-translational processing. If these proteins are produced by genetic engineering of *E. coli*, for example, the post-translational processing must often be accomplished using complex, *in vitro* chemical procedures which are cost-prohibitive for large-scale production applications. Even when peptides are recombinantly produced using mammalian hosts, it is often desirable to efficiently produce a precursor which is only later subjected to further modification.

One type of such further processing activity involves the specific amidation of the carboxy-terminal amino acid of a peptide or protein. Many naturally-occurring hormones and peptides contain such a modification, which is often essential if the protein is to be biologically active. An example is calcitonin, where the substitution of a non-amidated proline residue for the amidated proline of the native form results in a very significant reduction in biological activity. Other biological peptides requiring post-translational amidation for full activity include but are not limited to growth hormone releasing factor, other calcitonins, calcitonin gene-related peptide, secretin, Peptide YY and the like.

The polypeptide sequences for many important biological proteins which require amidation for maximal efficacy, may be manufactured, for example, by genetic engineering techniques. However, the important and sometimes essential carboxy terminal amidation must often be performed *in vitro*. It is desirable to avoid costly and cumbersome chemical amidation techniques at this point, and is therefore desirable to utilize an amidating enzyme to perform the specific amidation. The specific amidation of

the carboxy-terminal amino acid of a protein is frequently catalyzed by alpha-amidating enzymes.

Peptidylglycine α -amidating monooxygenase (PAM) catalyzes the conversion of a peptide substrate to an amidated peptide product. The conversion is a two-step reaction. PAM has two catalytic domains: peptidylglycine α -hydroxylating monooxygenase (PHM) catalyzes Step 1 (conversion of substrate to intermediate) and peptidylglycine α -hydroxyglycine α -amidating lyase (PAL) catalyzes Step 2 (conversion of intermediate to product). Full length PAM is bifunctional and catalyzes both steps. Step 2 may also be efficiently accomplished non-enzymatically if the intermediate is contacted with a Lewis base.

In nature, approximately 50% of peptide hormones and neurotransmitters are amidated by PAM in the foregoing manner. PAM activity has been recognized in numerous diverse species, PAM tends to have significant structural homology among species as diverse as rat, cow and frog. It is also known that PAM's function, substrate and cofactors are similar (frequently identical) across species. The substrate is a compound, often a peptide, having a glycine residue with a free carboxyl group. PAM-catalyzed amidation reactions are well known in the art. For example, one is described in detail in United States Patent 6,103,495 where a peptidylglycine α -amidating monooxygenase is used to catalyze the conversion of a glycine-extended salmon calcitonin precursor to authentic salmon calcitonin, amidated at its C-terminus (*i.e.*, having an amino group in place of the precursor's C-terminal glycine).

Sources of PAM and cell lines expressing PAM are known in the art. Large scale PAM-catalyzed production of amidated peptides requires a stable high-yield source of PAM for best cost-effectiveness. Additionally, because PAM is frequently utilized for the production of potential human pharmaceutical products, it is important that the system for PAM production (both the PAM-expressing cell line, and the culture media in which it is grown) introduce as few impurities as possible during PAM expression. Of particular concern, is minimizing the use of mammalian proteins in order to avoid the risk

of transmissible spongiform encephalopathies (TSEs). Mammalian protein growth factors in the culture media (undesirable from a TSE standpoint) may nonetheless be useful in aiding the survival and product expression of PAM-expressing cell lines. Thus, there is a need in the art for PAM-expressing (or PHM-expressing) cells that provide strong expression and show good stability and survival even in the absence of mammalian proteins that might otherwise be required in the media.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide PAM-expressing or PHM-expressing cells which are hardy enough to have good survival rates and good expression yields in media substantially devoid of the types of mammalian proteins and other impurities that could be problematic when the enzyme is later used in the manufacture of products (e.g. manufacture of amidated pharmaceutical products).

It is a further object of the invention to provide PAM-expressing or PHM-expressing cells that do not themselves co-express significant undesirable impurities.

It is a further object to provide PAM-expressing or PHM-expressing cells that provide good expression yields.

It is a further object to provide expression vectors useful in such cells.

It is a further object to provide good techniques for transfecting and selecting such cells.

It is a further object to provide high activity and high purity PAM or PHM for use in enzymatic reactions, and to thereby provide good enzymatic reactions and resulting amidated product.

These and other objects are provided by the inventions disclosed herein.

In one embodiment, the invention provides a CHO K1 cell transfected with an expression vector for expressing peptidylglycine alpha-amidating monooxygenase.

In another embodiment, the invention provides a recombinant expression vector having a coding region with nucleic acids encoding peptidylglycine alpha-amidating monooxygenase, operably linked to a control region including a ribosome binding site, a promoter and an SV40 enhancer upstream of said promoter.

In another embodiment, the invention provides a recombinant expression vector having a coding region with nucleic acids encoding peptidylglycine alpha-amidating monooxygenase, operably linked to a control region including a ribosome binding site and, a human metallothionin IIA promoter.

In another embodiment, the invention provides a method of preparing a cell line for the expression of peptidylglycine alpha-amidating monooxygenase, said method comprising the steps of:

- (A) Transfecting potential host cells in the presence of first, second and third expression vectors, wherein said first vector includes a coding region encoding a first selectable marker, wherein said second vector includes a coding region encoding a second selectable marker, wherein said third vector includes a coding region encoding peptidylglycine alpha-amidating monooxygenase, wherein the concentration ratio of said third vector to said first vector is at least 3:1, and wherein the concentration ratio of said third vector to said second vector is at least 3:1;
- (B) Subjecting the cells resulting from step (A) to selectable pressure to select cells that have been transfected with said first vector;
- (C) Subjecting the cells resulting from step (B) to selectable pressure to select cells that have been transfected with said second vector;
- (D) Subjecting the cells resulting from step (C) to limiting dilution and selecting cells expressing peptidylglycine alpha-amidating monooxygenase.

In another embodiment, the invention provides cell line UGL 73-26/M.

In another embodiment, the invention provides peptidylglycine alpha-amidating monooxygenase expressed by cell line UGL 73-26/M.

In another embodiment, the invention provides a method of purifying peptidylglycine alpha-amidating monooxygenase following expression and secretion into culture media, wherein said method includes the steps of :

- (A) subjecting an impure sample of peptidylglycine alpha-amidating monooxygenase to anion exchange chromatography wherein elution is isocratic;
- (B) subjecting the eluant of step (A) to hydrophobic interaction chromatography, wherein ammonium sulfate is not used and wherein elution is isocratic.

In another embodiment, the invention provides a CHO K1 cell transfected with an expression vector for expressing peptidylglycine alpha-hydroxylating monooxygenase.

In another embodiment, the invention provides a recombinant expression vector having a coding region with nucleic acids encoding peptidylglycine alpha- hydroxylating monooxygenase, operably linked to a control region including a ribosome binding site, a promoter and an SV40 enhancer upstream of said promoter.

In another embodiment, the invention provides a recombinant expression vector having a coding region with nucleic acids encoding peptidylglycine alpha-hydroxylating monooxygenase, operably linked to a control region including a ribosome binding site and, a human metallothionin IIA promoter.

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said third vector to said first vector is at least 3:1, and wherein the concentration ratio of said third vector to said second vector is at least 3:1;

(B) Subjecting the cells resulting from step (A) to selectable pressure to select cells that have been transfected with said first vector;

(C) Subjecting the cells resulting from step (B) to selectable pressure to select cells that have been transfected with said second vector;

(D) Subjecting the cells resulting from step (C) to limiting dilution and selecting cells expressing peptidylglycine alpha- hydroxylating monooxygenase.

In another embodiment, the invention provides a method of purifying peptidylglycine alpha- hydroxylating monooxygenase following expression and secretion into culture media, wherein said method includes the steps of:

(A) Subjecting an impure sample of peptidylglycine alpha- hydroxylating monooxygenase to anion exchange chromatography wherein elution is isocratic;

(B) Subjecting the eluant of step (A) to hydrophobic interaction chromatography, wherein ammonium sulfate is not used and wherein elution is isocratic.

The desired enzyme is expressed by the cells and cell lines of the invention, and in preferred embodiments purified in accordance with a purification techniques of the invention. Reactions are then performed in the presence of the enzyme starting with precursors which have a glycine residue, in free acid form and attached to a carbonyl group. Reaction conditions and co-factors are known in the art. Examples 1 and 2, herein, show a typical amidation reaction and purification of amidated product. Preferred amidated products result. When peptidyl alpha-hydroxylating monooxygenase is utilized, an intermediate results which requires further reaction with either a Lewis base or peptidyl alpha-hydroxy glycine alpha-amidating lyase to form the amidated product.

When enzyme-producing cell lines such as those discussed herein express and secrete enzyme into culture media, a series of steps may be followed, in certain embodiments, to

purify the enzyme. A harvesting step separates conditioned medium from cells. A first tangential flow filtration (concentration, diafiltration) removes low molecular weight components. An anion exchange chromatography step and a hydrophobic interaction chromatography step are utilized primarily for purification from protein impurities and medium components. A final tangential flow filtration, concentration and buffer exchange prior to virus filtration are desirably employed prior to storage. Improved anion exchange chromatography and hydrophobic interaction chromatography are claimed herein.

It has surprisingly been found that desirably stable CHO K1 cells may be used in the context of the invention despite the presence of an endogenous dihydrofolate reductase gene. The endogenous gene did not significantly interfere with methotrexate selection based on a selectable marker having a dihydrofolate reductase gene.

Co-transfecting the PAM gene with two selectable markers on independent vectors, as described herein, is believed to significantly increase the chances of co-amplification occurring at more desirable parts of the genome.

When an SV40 enhancer is used, it is understood that the enhancer may be used together with any transcriptional promoter including but not limited to the SV40 transcriptional promoter, the preferred metallothionin IIA promoter discussed herein and the like.

Peptidylglycine alpha-amidating monooxygenase expressed and purified in accordance with the present invention is substantially free of undesirable protease activity and is suitable for efficient alpha-amidation of substrate as described herein.

A preferred cell line discussed herein, UGL 73-26/M, showed a good longevity profile, and is hence believed to be particularly useful for scale-up where stability is of significant importance.

Likewise, the preferred purification techniques discussed herein provide scalable characteristics and significant product purity. Specifically, isocratic elution is used which

significantly simplifies fraction collection. Prior art use of ammonium sulfate in connection with hydrophobic interaction chromatography is desirably avoided because ammonium sulfate can cause precipitation and inactivation of the enzyme.

By analogy, it is expected that the inventions described herein will be beneficial with regard to peptidylglycine alpha-hydroxylating monooxygenase, as they have been beneficial with peptidylglycine alpha-amidating monooxygenase.

One or more of the preferences stated herein may be used in combination. For example, and not by way of limitation, preferred promoters may be used together with preferred enhancers and/or with preferred host cells.

When (A) PHM and (B) either PAL or a Lewis base, are used for amidation in lieu of PAM, the PHM and PAL (where used) may be obtained in a number of ways. Some are set forth below.

PHM

Express naturally occurring forms of the PAM gene that when expressed contain only the PHM activity

One enzyme derived from frog is a naturally occurring PHM enzyme. See Mizuno et al (1986), "Peptide C-Terminal α -Amidating Enzyme Purified to Homogeneity From Xenopus laevis Skin" *Biochem Biophys. Res. Commun.*, 137 (3) 984-991, which was later found to be a PHM rather than full-length PAM. See Suzuki et al (1990), *EMBO* 9 (13) 4259-4265.

Express a full length PAM and cleave

A specific protease could be used to cleave the PAM enzyme at a site between the PHM activity and the PAL activity, for example a dibasic cleavage site. The PHM catalytic domain can then be obtained by purification. For example, Ouafik et al, (1992) "The Multifunctional Peptidylglycine α -Amidating Monooxygenase Gene: Exon/Intron Organization of Catalytic, Processing, and Routing Domains" *Molecular Endocrinology*

6 (10) 1571-1584 describes the location of the two catalytic domains in a rat-derived PAM, and notes that "endoproteolytic cleavage at a paired basic site can separate the two catalytic domains." See also Eipper et al (1993), "Peptidylglycine α -Amidating Monooxygenase: A Multifunctional Protein With Catalytic, Processing, and Routing Domains" *Protein Science* 2, 489-497.

Introduce a stop codon in PAM or a point mutation that alters the reading frame

As an alternative, translation stop codons (TAA, TAG, TGA) can be introduced in any PAM cDNA from any species between the two functional domains of PAM (PHM and PAL), or a point mutation in such a location could be introduced to alter the reading frame.

Reengineer an expression vector with only a PHM cDNA

Using PCR, a truncated PAM gene encoding only the PHM domain could be synthesized and put into an expression vector downstream of a promoter or enhancer/promoter sequence.

Set forth below are additional references for different types of PAM enzyme and its catalytic domains:

Mizuno, K. et al., (1987) "Cloning and sequence of cDNA encoding a peptide C-terminal α -amidating enzyme" from *Xenopus Laevis Biochem Biophys. Res. Commun.*, 148 (2) 546-552.

Ohsuye, K., et al., (1988) "Cloning of a cDNA encoding a new C-terminal α -amidating enzyme having a putative membrane-spanning domain" from *Xenopus Laevis Biochem. Biophys. Res. Commun.*, 150 (3) 1275-1281.

Koljekar, A.S., et al., (1997) "Peptidylglycine α -amidating hydroxylating monooxygenase: active site residues, disulfide linkages and tow-domain model of the catalytic core", *Biochemistry* 36:13901-13909.

Koljekar, A.S., et al., (2002) "Essential features of the catalytic core of Peptidylglycine α -hydroxyglycine α -amidating lyase", *Biochemistry*, 41:12384-12394.

Bertelsen, A.H., et al., (1990) "Cloning and characterization of two alternatively spliced rat α -amidating enzyme cDNAs from rat medullary thyroid carcinoma", *Arch. Biochem. Biophys.*, 279 (1) 87-96.

Jimenez N., et al., (2003) "Androgen-independent expression of adrenomedullin and peptidylglycine α -amidating monooxygenase in human prostatic carcinoma", *Molecular Carcinogenesis* 35: 14-24.

PAL

Express naturally occurring forms of PAL

The two catalytic domains of PAM are encoded by separate genes in *Drosophila* and in cnidarians (sea anemone). Therefore, the gene encoding PAL from these species can be expressed when put into an expression vector downstream of a promoter or enhancer/promoter sequence. See Kolhekar, A.S., et al. (1997) "Neuropeptide Amidation in *Drosophila*: Separate Genes Encode the Two Enzymes Catalyzing Amidation" *J. Neuroscience* 17(4):1363-1376.

Express a full length PAM and cleave

Full length bifunctional PAM could be expressed, and cleavage at a specific protease site can be performed in between the PHM and PAL domains and/or within the PHM domain to inactivate the PHM. Any suitably specific protease could be used to disassociate the PHM activity from the PAL activity, for example, a dibasic cleavage site. The PAL activity/protein can be further purified. A similar procedure and a location for the dibasic cleavage site are described above in connection with obtaining PHM.

Reengineer an expression vector with only a PAL cDNA

Using PCR a truncated PAM gene encoding only the PAL domain could be synthesized and put into an expression vector downstream of a promoter or enhancer/promoter sequence.

Other features and advantages of the present invention will become apparent from the following description of the invention which refers to the accompanying figures, charts, tables, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a plasmid map of pHS1.

Fig. 2 is a plasmid map of pUC8.

Fig. 3 is a plasmid map of pSV402MT.

Fig. 4 is a derivation of α -AE cDNA Gene Sequence.

Fig. 5 is a plasmid map of pAE73.

Fig. 6 is a 73-26 suspension adaptation scheme.

Fig. 7 is a dissolved oxygen concentration and α -AE productivity in spinner flasks.

Fig. 8 is an effect of spinner volume on DO and α -AE productivity.

Fig. 9 is a spinner culture pH of UGL 73-26/M.

Fig. 10 is a pH profile of stirred tank bioreactors and spinner flasks without pH control.

Fig. 11 is a pH effect on UGL 73-26/M α -AE productivity in stirred tank bioreactors.

Fig. 12 is a SDS PAGE of purified PAM enzyme.

Fig. 13 is a densitometry scan of SDS PAGE gel and calculation of percent peak areas.

Fig. 14 is a process flow diagram for production of α -AE.

Fig. 15 is an inoculum scheme for UGL 73-26/M.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

A PAM-expressing cell line in accordance with the invention (internally designated UGL 73-26/M MWCB 00) was deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas Virginia, 20110-2209, U.S.A., on or about June 10, 2005, pursuant to the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure. The ATCC accession number is PTA 6784. This deposited cell line is subject to the Regulations promulgated under this Treaty, and samples will be made available at the time, and under the conditions required by, the Treaty, and in compliance with the patent laws and regulations of the Treaty signatories. For example, upon issuance of a U.S. patent based on this application or any other U.S. application claiming priority hereof or making reference hereto, all restrictions upon availability of the deposited material will be irrevocably removed to the extent required by the Budapest Treaty or by 35 U.S.C. §112.

The construction of a PAM-expressing vector of the invention is described in detail *infra*. In the plasmid maps, herein, The region marked "alpha amidating enzyme" (see e.g., Figure 5, the plasmid map of pAE 73) has the nucleotide sequence set forth as SEQ ID NO. 1 appended hereto, and encodes a 715 amino acid primary translation product set forth as SEQ ID NO. 2 appended hereto. That translation product includes a signal peptide at amino acids 1-25, a pro-region at 26-41, and a mature peptidylglycine alpha-amidating monooxygenase at 42-715. Most of the enzyme expressed and purified in accordance with the invention continues to include the pro-region, and has proven to be very effective.

Detailed below are examples of a preferred PAM-expressing cell line, its fermentation, process development, purification and use.

As discussed in more detail, *infra*, a PAM gene was cloned into the pSV402MT expression vector to create the PAM expression plasmid pAE73. CHO K1 cells were transfected with pAE73 DNA. Following a dual selection process and limiting dilution cloning a CHO cell line was further amplified with increasing concentrations of methotrexate. The adherent cell line 73-26 was converted to a selection free, serum-free suspension culture, UGL 73-26/M. A UGL 73-26/M MCB and MWCB were created at BioReliance Corp. Both the MCB and MWCB have been fully characterized and are deemed acceptable for production of PAM in a GMP facility. The UGL 73-26/M cell line has been adapted to grow in a stirred tank bioreactor. Fermentation development has led to defining an inoculum process that takes 14 days in spinner flasks. The fermentation/cell culture phase is a 17 day process. The key parameters of the fermentation process are as follows: glucose addition (2 g/L) on days 5, 10 and 14, dissolved oxygen concentration $\geq 70\%$, the pH of the fermentation to reach its natural set point, and a protein-free, non-animal sourced tissue culture media, Sigma C5467, supplemented with glutamine to a final concentration of 2 mM. A simple and robust downstream purification process was developed for the PAM from C5467 conditioned CHO medium. A four-fold increase in purity and 40% overall recovery of active enzyme are routinely achieved after purification. The process utilizes conventional filtration systems and bioprocess chromatography resins that are amenable to scale-up to the manufacturing level. The entire cell culture fermentation and downstream purification processes have been scaled to a 10 L pilot plant/manufacturing level. The process as described should be amenable to a validatable manufacturing process for the production of large quantities of PAM.

A CHO cell line was developed that expresses large amounts of PAM, UGL 73-26/M. The cell culture process developed for UGL 73-26/M was in a non-animal protein sourced media. A simple batch cell culture process was preferred. The objective of the

purification scheme was to develop a robust and scalable process with good yields of catalytically active enzyme.

Vector Construction

The creation of the expression vector used to generate the cell line, pAE73 was based on results from the work of Friedman et al. Bio/Technology 7:359-362 (1989). Following the process described in this article, we proceeded to create the pAE73 expression vectors for testing. The major components of each vector are the human metallothionein IIA (hMTIIA) promoter (located in pHS1, received from M. Karin), the SV40 enhancer (derived from pSV40, ATCC), and the α -amidating enzyme gene described herein.

Derivation of pAE73

pHS1

The starting plasmid used in the creation of pAE73 was the plasmid pHS1 (Figure 1), a gift from M. Karin at UCSD in February 1990. pHS1 was derived in Dr. Karin's laboratory by splicing into pUC8 (Figure 2) the human metallothionein IIA promoter. The 846 bp Hind III/Bam H1 fragment was cloned into the Hind III-Bam H1 site of the pUC8 multiple cloning site (MCS). The resulting plasmid, pHS1, has the human metallothionein IIA promoter inserted in the multiple cloning site.

pSV401MT and pSV402MT

pHS1 was converted into the expression vector pSV401MT or pSV402MT (Figure 3) by inserting the SV40 enhancer upstream of the human metallothionein promoter. The SV40 DNA fragment was prepared by digesting pSV40 plasmid DNA with Hind III. The 1167 bp Hind III fragment was cloned into the Hind III site of pHS1. The SV40 enhancer sits asymmetrically within the Hind III DNA fragment thus locating the enhancer either close to or far away from the hMTIIA promoter. The orientation of the Bgl I site within the SV40 enhancer relative to that in the lac Z gene in the original plasmid determines the plasmid designation. pSV401MT has the SV40 enhancer far away from the hMTIIA

promoter and pSV402MT has the SV40 enhancer close to the hMTIIA promoter. pSV402MT was chosen for further vector construction.

pAE 73

pAE73 was prepared by cloning the alpha-amidating enzyme gene (SEQ ID NO 1) into the Bam H1 site of pSV402MT. The 2870 bp α -AE gene fragment was isolated following digestion of pAE64 with Bgl 1 and Bam H1 DNA restriction endonucleases. The pAE64 plasmid contains a PAM gene downstream of the SV40 promoter/enhancer that has been modified to express a soluble 75 kDa PAM protein. This PAM DNA sequence has been used in another CHO expressing cell line, UGL B3/A1-7, to express the 75 kDa PAM protein. Derivation of the PAM gene sequence is shown in Figure 4.

The 2870 bp DNA fragment was purified and the Bgl 1 end of the fragment modified with two complimentary DNA oligonucleotide fragments (AE96/AE97) that converted the Bgl 1 end of the fragment to a new Bam H1 end.

AE96 (+) 23	5'	GATCCATCGATCGCACTAGTGCC	3'
AE97 (-) 16	5'	ACTAGTGCGATCGATG	3'

The modified PAM DNA fragment was cloned into the Bam H1 site of the expression vector, pSV402MT. The PAM DNA fragment with two Bam H1 ends could ligate into the expression vector in either the sense or antisense orientation, therefore the orientation of the PAM gene was determined by the restriction digest mapping of the plasmid with Eco R1. An Eco R1 site in both the PAM gene and the expression vector allows for a simple analysis of plasmid DNA. The plasmid with the PAM in the correct orientation was designated as pAE73, and it's plasmid map is shown in Figure 5

Creation of an α -Amidating Enzyme Expressing Cell Line

Transfection and Cloning

Three plasmids were used in the transformation of CHO K1 cells: pAE73, pSV₂neo and

pSV₂*dhfr*. The plasmids pSV₂neo and pSV₂*dhfr* (both obtained from the ATCC) were used because incorporation of these plasmids by transfected cells allows for easy selection of transformed cell lines. The plasmid pSV₂*dhfr* was also specifically chosen because incorporation of the SV40 promoter/*dhfr* DNA into the CHO genome allows for selective amplification of that gene and other proximal genes. The gene to be co-amplified was the α -amidating enzyme gene carried in the plasmid pAE73. CHO K1 cells were transformed by calcium phosphate precipitation of plasmid DNA. The plasmids were transfected into the cells in a ratio of 10:1:1, pAE73:pSV₂neo:pSV₂*dhfr*. 20 μ g pAE73 was added per 100 mm dish. Two days after the transfection, the cells were grown under selective pressure in a medium containing 250 mg/L G418 to allow for only transformed cells to survive. CHO cell growth in this medium would require stable incorporation of the pSV₂neo plasmid. Methotrexate was added to the growth medium 27 days after transfection, once stable growth was established in G418 selective media. The G418 pools of transformed cells were grown in media containing 100 nM, 500 nM, 1 μ M or 5 μ M methotrexate and 250 mg/L G418. Two weeks later (six weeks post transfection) isolated foci of cells could be established from cells grown in 5 μ M methotrexate + G418 media by cloning cylinders. An attempt was made to establish cell lines from twenty-five foci by this method, but cells from only two foci grew after transfer and this technique was abandoned. Limiting dilution cloning, 0.5 cells/well, was initiated at the same time from cells grown in media containing 1 μ M methotrexate + G418. Transformed cells grown in lower concentrations of methotrexate were abandoned. Three weeks after starting limiting dilution cloning isolates were transferred to 24-well plates and then to 100 mm dishes for expanded growth within 2-3 days. Ten weeks after initial transfection of CHO K1 cells, one of the isolates designated 73-26 was established. The cell line was cryopreserved and α -amidating enzyme expression assessed. 73-26 was one of the best producing cell lines at this juncture producing 6953 U/10⁶ cells/day.

Amplification of Cell Line 73-26

The primary clone of 73-26 was passaged to yield an established cell line maintained in media containing 1 μ M methotrexate + G418. The purpose of choosing the pSV₂*dhfr* plasmid for transfection was not only because it provided a second selection method to identify transformed cell lines but because it has been widely established that the *dhfr* mini gene is amplified when cells are grown at high concentrations of methotrexate. Unigene Laboratories had previous experience with cell lines transformed with this plasmid showing that concentrations of 20-50 μ M methotrexate may be required to elicit the maximum production of α -amidating enzyme. The α -amidating enzyme cell line 73-26 was split directly into media containing 1 μ M, 20 μ M, or 50 μ M methotrexate. All media also contained G418 as a second method of selection. The progression of α -amidating enzyme expression of this cell line at various time intervals is shown in Table 1.

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Table 1 Amplification and Expansion of CHO Cell Line 73-26

Date	Cell Line	Methotrexate (μ M)	α -AE (U/10 ⁶ cells/Day)	Relative Activity to B3/A1-7 Control	Comments
08/19/1991					cell line switched to media with increased concentrations of methotrexate
09/26/1991	73-26	1	15138		
	73-26	20	ND		cell line growth slow
	73-26	50	ND		cell line growth slow
10/28/1991	73-26	1	25000		
	73-26	20	6923		
	73-26	50	114162		
	73-26				
11/21- 22/1991	B3-A1/7		5714/4087		
	73-26	1	73099/30065	16.5	
	73-26	20	55925/7570	5.8	
	73-26	50	22055/40937	7.0	
11/22/1991, 12/10/1991	B3-A1/7		3250/4087		
	73-26	1	15277	4.7	
	73-26	20	41898	12.9	
	73-26	50	96941	29.8	
04/26/1992	B3-A1/7		4692		
	73-26	1	22934/26340	5.2	
	73-26	20	24935/17237	4.5	
	73-26	50	47088/78462	13.4	

ND = Not Determined

The data in Table 1 show the increased expression levels of α -amidating enzyme in this cell line as compared to that of the previously developed B3/A1-7 cell line. Co-amplification of α -amidating enzyme gene and the *dhfr* gene is apparent by the cell line's ability to express more enzyme with continued subculture at increased concentrations of methotrexate. The cells grown in the presence of 20 μ M or 50 μ M methotrexate had the best expression levels after 3-4 months of selective amplification. When continuously subcultured for several months, the expression levels of α -amidating enzyme decreased. Early frozen stocks of this cell line were therefore used for prepared suspension adapted cell lines.

Characterization of a Suspension Adapted α -Amidating Enzyme Expressing Cell Line 73-26

The ultimate goal in developing this cell line was to develop a process that was stable without the selective pressure of methotrexate and G418. Another desired attribute of the cell line was that it should be grown to relatively high cell densities in a suspension culture. The method used to achieve those goals is described below.

Preparation of Suspension Adapted, Selection-Free Cell Lines

Preparation of selection-free suspension adapted cells required that UGL 73-26 adherent cells be weaned off of serum, methotrexate and G418. A multi prong attack, Figure 5, was developed to accomplish this task; a matrix of removal was established with the ultimate goal of adding the least number of cell doubling on the final cell line as possible. An early freeze, 12/16/91, of the 73-26 20 μ M methotrexate cell line was thawed to establish a suspension adapted, serum free, selection free cell line for future developmental studies. Although the PAM activity in 50 μ M methotrexate was somewhat higher attempts to re-establish an actively growing 73-26 50 μ M cell line were unsuccessful. The α -amidating enzyme productivity of the cell line was assessed prior to initiating the suspension adaptation protocol, Table 2.

Table 2 Productivity of 73-26 20 μ M

Cell line	α -AE (U/10 ⁶ cells/Day)
CHO K1	0
B3/A1-7	22,555
73-26	446,625

The PAM activity of this cell line prior to adapting it to suspension culture had 20-fold greater activity on a per cell basis than the B3/A1-7 control. The enzyme activity for both the control cell line and 73-26 were 5-10 times that observed in the original testing just prior to when the cell line was frozen, 12/16/91 (Table 1).

Four new suspension adapted, serum free cell lines were generated as shown in Figure 6. The cell lines, designated as 73-26/K-N were cryopreserved following a 37 day process.

α -Amidating Enzyme Production of Suspension Adapted 73-26

Banked cells from the new 73-26 cell lines (73-26/K, 73-26/L, 73-26/M, 73-26/N) were thawed and grown in spinner flasks. The cell lines were maintained by passaging the cells three times per week in Ex-Cell 301 media. At periodic intervals a complete medium exchange was done and a 24 h assessment of enzyme productivity evaluated by the α -AE assay. The cell lines were maintained for up to 80 days in culture. The semi-continuous batch process for the previous cell line, B3/A1-7, was a 40-day process and we chose to evaluate the cell line production stability at twice the length of this procedure. The data from this study are shown in Table 3. During the course of this study cell line 73-26/L was terminated between day 22 and day 40, all other cell lines were actively growing at the end of the 80-day period.

Table 3 Enzyme Productivity of Suspension Adapted Cell Lines 73-26/K-N

Cell line	Days in Culture	α -AE Activity (U/10 ⁶ cells/Day)	Actual or Mean α -AE (U/10 ⁶ cells/Day)	Relative Activity to B3/A1-7 [*]	
B3/A1-7	15	4907	5068		
	15	5778			
	17	4520			
	42	15697			15697
	60	3773			3820
	60	3867			
	70	12677			12677
	80	14246			14246
73-26/K	20	16872	11836	2.34	
	20	10778			
	22	7859			
	40	8274	11609	0.74	
	47	14944			
	60	8616	5827.5	1.53	
	60	3039			
	70	10790	10790	0.85	
	80	1933	1933	0.14	
73-26/L	20	10163	8731	1.72	
	20	7076			
	22	8955			
Cell line	Days in Culture	α -AE Activity (U/10 ⁶ cells/Day)	Actual or Mean α -AE (U/10 ⁶ cells/Day)	Relative Activity to B3/A1-7 [*]	
73-26/M	20	14728	9954	1.97	
	20	8910			
	22	6223			
	40	44882	36391	2.32	
	47	27900			
	60	24844	23580	6.17	
	60	22316			
	70	46457	46457	3.66	
	80	41246	41246	2.90	
73-26/N	20	10173	13686	2.70	
	20	15852			
	22	15033			
	40	27368	26243	1.67	
	47	25117			
	60	16175	22123	5.79	
	60	28071			
	70	39082	39082	3.08	
	80	6915	6915	0.49	

*Note: Days in culture testing periods compared are results from 15-22, 40-47, 60, 70 and 80.

Although all cell lines expressed significantly more α -amidating enzyme than B3/A1-7 at the early intervals, <40 days, only 73-26/M and 73-26/N were still producing large quantities of enzyme after 70 days. UGL 73-26/M was chosen for further development because it consistently produced more α -amidating enzyme over a 80 day period. The other three cell lines either stopped growing before the end of the 80 day test period or their productivity/cell/day waned over the last 20 days.

PreBanking, Cell Banking Characterization Studies of CHO K1, UGL 73-26, UGL 73-26/M MCB and UGL 73-26/M MWCB00

Characterization studies were performed on the host cell line (CHO K1), a progenitor cell line (73-26) and the UGL 73-26/M seed bank. The 40 vial UGL 73-26/M seed bank was prepared on 2/4/98. Each vial of the seed bank contains 4×10^6 cells/mL in 90% Ex-Cell 301/10% DMSO. All studies performed were evaluated as consistent with the results required of a CHO cell line to be made into a MCB and MWCB, see Table 4.

The Master cell bank UGL73-26/M cell line was made at BioReliance Corp. Several of the 118 MCB vials were used for a full characterization of the cell line. Results from all studies of the UGL 73-26/M MCB are consistent with a cell line of CHO origin and negative for infectious agents. A vial of the UGL 73-26/M MCB was used to create a 238 vial manufacturer's working cell bank, UGL 73-26/M MWCB00, at BioReliance Corp. All results from studies of the UGL 73-26/M MWCB00 are consistent with cell line of CHO origin and negative for infectious agents.

Table 4 Summary of CHO Cell Characterization Studies

Cell Banking Phase	Test Article	Test	Results	Lab Completion Date	Report #
PreBanking	CHO K1	Transmission Electron Microscopy	Negative - No cells with A-type and budding C-type particles	8/23/1999	AA18UW.013000.PAI
	UGL 73-26	Transmission Electron Microscopy	Negative - No cells with A-type and budding C-type particles	08/17/1999	AA18PK.013000.PAI
	UGL 73-26/M Seed Bank	Sterility	Satisfactory	02/17/1999	AA11VS.510000.BSV
		Mycoplasma	Negative	03/02/1999	AA11VS.102003.BSV
		MVM - PCR Assay	Negative	02/09/1999	AA11VS.105026.BSV
		Transmission Electron Microscopy	Negative - 3 cells with A-type and budding C-type particles	03/05/1999	AA11VS.013000.PAI
		Murine Retrovirus - Co-Cultivation with Mink Lung Cells	Negative	09/14/1999	AA18UH.011001.BSV

Table 4 continued

		Repeat of Transmission Electron Microscopy	Negative - No cells with A-type and budding C-type particles	09/15/1999	AA18UH.013000.PAI
Master Cell Bank	UGL 73-26/M MCB	Sterility	Satisfactory	12/21/1999	AA24KX.510000.BSV
		Mycoplasma	Negative	01/03/2000	AA24KX.102003.BSV
		Isozyme	Consistent with Chinese hamster origin	02/09/2000	AA24KE.380001.BSV
		In Vitro adventitious agents	Negative	02/09/2000	AA24KE.003000.BSV
		In Vivo Inapparent Viruses	Negative	02/11/2000	AA24KE.005002.BSV
		In Vivo adventitious agents	Negative	02/15/2000	AA24KE.004005.BSV
		Transmission Electron Microscopy	Negative - No cells with A-type and budding C-type particles	02/08/2000	AA24KE.013000.PAI
		Reverse Transcriptase	Non-informative	01/15/2000	AA24KE.002000.BSV

Table 4 continued						
		Murine Retrovirus - Co-Cultivation with Mink Lung Cells	Negative	02/01/2000		AA24KE.011001.BSV
		Murine Retrovirus - Co-Cultivation with RD Cells	Negative	02/01/2000		AA24KE.011002.BSV
		MVM - PCR Assay	Negative	02/03/2000		AA24KE.105026.BSV
		MAP	Negative	02/25/2000		AA24KE.004000.BSV
		In Vitro Bovine Virus	Negative	02/04/2000		AA24KE.032004.BSV
		In Vitro Porcine Parvovirus	Negative	02/04/2000		AA24KE.033004.BSV
Manufacturer's Working Cell Bank	UGL 73-26/M MWCB00	Sterility	Satisfactory	03/24/2000		AA27LH.510000.BSV
		Mycoplasma	Negative	04/07/2000		AA27LH.102003.BSV
		In Vitro adventitious agents	Negative	04/26/2000		AA27LH.003031.BSV
		In Vivo Inapparent Viruses	Negative	05/16/2000		AA27LH.005002.BSV
		Isozyme	Consistent with Chinese hamster origin	04/21/2000		AA27LH.380001.BSV

Process Development Studies of UGL 73-26/M in Spinner Flasks and Stirred Tank Bioreactors

Since preliminary experiments had indicated that the productivity of the cell line could not be maintained consistently over a large number of generations, a batch fermentation protocol was investigated. A batch protocol also offers the advantages of ease of scheduling, easier scalability and less drastic consequences of batch failure. Fermentation process development for UGL 73-26/M required investigation of a number of parameters that could influence the cellular productivity of the cell line. The fermentation development detailed below shows pivotal studies investigating dissolved oxygen (DO), pH and media supplementation. Fermentation parameters not studied were impeller RPM and fermentation temperature.

Effect of Dissolved Oxygen on α -Amidating Enzyme Expression

An experiment was performed to examine the effect of dissolved oxygen concentration of the CHO culture media in a spinner flask. Spinner flasks were seeded with 0.1×10^6 cells/mL. Two spinner flasks contained 150 mL of media in a 250 mL Techne spinner flask, whereas the third spinner flask contained 250 mL of media. Daily measurements of dissolved oxygen were taken on all spinner flasks. Aliquots of clarified conditioned media were taken daily for assessment of α -AE productivity by the α -AE assay. Two culture media were used in this study, a low protein CHO media (C1707, Sigma-Aldrich) and a protein-free CHO media (C5467, Sigma-Aldrich). The direct comparison of UGL 73-26/M grown in the two media is shown in Figure 7. The dissolved oxygen level in the two cultures was markedly different. The cells grown in 150 mL of C1707 produced less total enzyme/mL than those cells grown in an equivalent volume of C5467 culture media. Although the initial dissolved oxygen concentration of the media were the same, ~80% DO, the dissolved oxygen concentration of the C5467 media culture never went below 50%. The DO content of the C1707 culture was less than 50% by Day 9 and the peak activity of the culture was two days later. These data indicate that there may be a correlation between DO content/cell viability/ α -AE productivity.

Spinner volume was also investigated as a potential critical parameter. Because the

spinner flask is not maintained at a constant dissolved oxygen concentration, the culture's ability to maintain dissolved oxygen is relative to the cultures surface:volume ratio. Increasing the culture volume of a spinner appreciably alters the surface:volume ratio. Identical cultures were started except that the culture volume in one spinner was 150 mL (5467s) and the other was 250 mL (5467sa). The effect of changes in surface:volume ratio on %DO and α -AE productivity are shown in Figure 8.

The data shown in Figure 8 show that the productivity of the culture is greatly affected by the dissolved O₂. Whether the dissolved O₂ concentration decreases because of the utilization by the culture or because of the effect from differences in surface:volume ratio; when the DO concentration is less than 50% the culture ceases to express more α -amidating enzyme within 48-72 h. The dissolved O₂ in the bioreactor should be maintained at 70% to reflect the dissolved oxygen concentration during the phase of the culture where the cells are the most healthy and productive.

Effect of Media on α -Amidating Enzyme Expression

As parts of studies either designed to facilitate selection of the most appropriate choice of growth medium for UGL 73-26/M or while defining bioreactor growth conditions numerous spinner cultures were initiated with two CHO culture media, C1707 and C5467 (Sigma-Aldrich). Both culture media are defined, C1707 is a low protein media with transferrin added to the media and C5467 is a non-animal protein media. Both media, when purchased, required addition of L-glutamine to a final concentration of 2 mM. Table 5 below tabulates the data from a large sampling of spinner flasks with either of the two media.

Table 5 Summary Of Spinner Cultures Data With C1707 And C5467 CHO Culture Media.

Media Formulation	n=		Peak Enzyme Activity (units/mL)	Day of Peak Activity	Peak Cell Density (10 ⁶ cells/mL)	Day of Peak Cell Density
C5467	21	Mean	363,366	15	1.48	11
		SEM	51,708	0.5	0.11	0.6
C1707	12	Mean	322,691	14	1.92	10
		SEM	86,045	2.9	0.66	3.0

The data in Table 5 show no substantial difference in enzyme productivity of UGL 73-26/M cell line in either media. Sigma CHO culture media, C5467, may be more regulatory compliant media choice concerning future regulatory guidelines in both the US and the European Union because it contains no animal sourced media components. Sigma CHO culture media, C5467, is the media of choice for future studies.

Effect of Glucose on α -Amidating Enzyme Expression

Studies investigating the nutrient status of a UGL73-26/M batch culture revealed that 4-5 days after the initiation of the culture the glucose concentration in the media was approximately 50% (2 g/L) that of virgin media. A thorough study of the effect of glucose supplementation on enzyme productivity was undertaken as a series of spinner flask experiments where additional glucose is added to the culture. Spinner flasks were supplemented with 2 g/L glucose 1 to 3 times at specific time intervals. The effect of glucose addition on α -AE productivity is shown in Table 6.

Table 6 Glucose Supplementation – Relative Enzyme Expression

Day of Glucose Addition	Peak Activity - % of Control					Average
	00108	00110	2130-D-1001 Fairfield	2131-D-1001	2131-D-1002	
None	100	100	100	100	100	-
5	151		160			156
5, 10	235	162	144	111	65	143
5, 10,15	197		192			195
8, 15	179		149			164

Table 7 Glucose Supplementation – Day Of Peak Enzyme Expression

Day of Glucose Addition	Day of Peak Activity					Average
	00108	00110	2130-D-1001 Fairfield	2131-D-1001	2131-D-1002	
None	15	13	12	13	13	13
5	17					17
5, 10	19	19	13	23	17	18
5, 10,15	19		15			17
8, 15	19		16			18

Addition of glucose to a batch culture of UGL 73-26/M increased the maximum productivity of the culture by 50-100% depending on the glucose addition regimen. The spinner flasks that were supplemented with glucose on days 5, 10 and 15 appear to express the most enzyme as compared to non-supplemented controls. The average relative productivity of these cultures was 195% of control. Other supplemented cultures were only slightly less productive (143-164%) than those supplemented three times. All cultures that were supplemented with additional glucose showed delayed peak enzyme concentrations in the media. Peak enzyme concentrations were observed on days 17/18 for glucose supplemented cultures versus day 13 in the control non-supplemented cultures, Table 7.

Effect of pH on α -Amidating Enzyme Expression

The pH of the conditioned media one of the process variables that can be controlled in a stirred tank bioreactor which can not be addressed in a spinner flask. In a spinner flask, the pH of the culture is allowed to drop as the media components are consumed and as cellular byproducts are produced. The pH profile of two 250 mL spinner flasks of UGL 73-26/M containing either 150 mL (5467s) or 250 mL (5467sa) are shown below in Figure 9. The pH of the culture decreased during the first portion of the batch and then rose during the last portion of the culture. The decrease in pH is likely due to increased lactate concentration in the culture at the beginning of the culture. The rise in the pH at

the end of the culture, is due to the cells catabolizing the lactate as a carbon source. The productivity of the culture is unaffected by the decrease in pH (Figure 8).

Several (n=5) 6-10 L stirred tank bioreactors were run without pH control to mimic conditions in a spinner flask. CHO cells in the stirred tank bioreactors were grown in C5467 media, at 70% DO and at 37°C. The average pH profile of the conditioned media in the bioreactor is similar to that observed in the two spinner flasks, Figure 10. The dissolved oxygen concentration in the bioreactor was maintained at 70% and the temperature at 37°C.

In addition to the bioreactor runs described above without pH control, a series of bioreactors were run where the pH of the reactor was allowed to free fall to the set pH point (between 7.0-7.4). At the time that the desired pH was reached, it was maintained at that pH for the duration of the fermentation. CHO cells in the stirred tank bioreactors were grown in C5467 media, at 70% DO and 37°C. The effect of maintenance of pH control on α -AE productivity in a stirred tank bioreactor is shown in Figure 11.

Figure 11 shows the average α -AE activity in the bioreactor for each day throughout the run for those runs when a pH set point was not initiated. The data from individual runs when the pH set point was set only after the pH was within 0.5 pH units of the set point are also shown in Figure 11. There is a clear correlation between culture pH and enzyme activity. The activity profile for the bioreactor run with no pH set point is superior for all runs during the initial 16 days of culture. The lone exception was the profile for the run where the pH set point was 7.1. The difference was probably because that culture lasted longer than the other runs. Peak α -AE activity for the average no pH set point studies was 280,707 units/mL on day 16.

There was no effect of pH on viable cell density (data not shown). The cultures grew to a peak viable cell density of $1.0-1.5 \times 10^6$ cells per mL. Maximum cell density was reached 8 to 10 days after the bioreactor was inoculated. There was no effect of pH on cell viability except for the one culture run at pH 7.1 (data not shown). Cell culture viability was greater than 50% viable for 15 or more days in culture. The one bioreactor

that was run with a pH set point of 7.1 maintained >50% viable cells for 20 days in culture.

Summary of Research and Development Fermentation Parameters for UGL 73-26/M

Following the establishment of the α -AE expressing cell line UGL 73-26/M, a series of experiments were done to define critical parameters for optimal expression of the α -amidating enzyme. It was determined that an animal protein-free media (Sigma, C5467) supported high levels of expression of the enzyme. The CHO cells could be grown as a batch culture in C5467 media supplemented with L-glutamine at a final concentration of 2 mM. However, the culture requires D-glucose supplementation to further boost the productivity of the culture. Optimum glucose supplementation was 2 g/L on days 5, 10 and 15. The DO concentration of the bioreactor should be maintained at 70% and the pH of the bioreactor allowed to adjust to the pH that is a function of the buffering capacity of the media and the metabolism of the media components by the CHO cells.

Downstream Purification Development UGL 73-26/M

The following section summarizes our downstream purification development for PAM produced using the UGL 73-26/M clone. Only representative experiments, which provide a logical progression of the process development work, are included in the summary. A brief description of each step is provided that describes the function of that step in the purification process. Purification runs were analyzed using SDS-PAGE (constant protein and constant units), PAM activity assay and Bradford protein assay. All of the experiments were completed prior to or shortly after the technology transfer to the Boonton, NJ pilot facility. The methods described herein were used to manufacture PAM batches 1330-D-1003, 1330-D-1004, 1330-D-1005, 1330-D-1006, 1330-1009 and 1330-1010.

Tangential Flow Filtration No. 1 (TFF 1)

Step Description: The TFF 1 step is utilized to concentrate and diafilter the conditioned

CHO cell media prior to chromatography. The conditioned media is concentrated and the conductivity is decreased by diafiltration to facilitate binding to the anion exchange column. The TFF 1 step employs a Pellicon 2 Module fitted with a regenerated cellulose PLCTK 30 kDa membrane (Millipore).

Research and Development: The initial investigation of this step focused on whether or not TFF would be necessary prior to chromatography. Reducing the conductivity of the conditioned CHO media by dilution with water was investigated as an alternative to TFF. Failure to dilute the conditioned media prior to chromatography caused a large portion of the PAM activity to reside in the flow through following loading. Decreasing the conductivity of the conditioned media to approximately 5 mS minimized the amount of PAM activity detected in the flow through. However, two problems were identified when the conditioned media was loaded directly onto the anion exchange column. One of the medium components irreversibly bound to the column and caused severe discoloration of the resin. Another medium component that exhibited enormous UV absorbance characteristics co-eluted with the PAM activity following gradient elution.

Conditioned CHO cell medium (C5467) was diluted with water to reduce the conductivity to approximately 5 mS and loaded directly onto a Q-Sepharose FF column (Pharmacia) equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0. The column was subjected to a linear gradient from 100% A (50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0) to 68% B (50 mM TRIS, 475 mM NaCl, 0.001% TX-100 pH 8.0) over 60 minutes. A large dark colored band appeared at the top of the column immediately following loading and remained throughout the chromatography run. The column was cleaned with 2 M NaCl and sanitized with 1.0 M NaOH. The cleaning and sanitization procedures failed to remove the dark band from the column. Subsequently, the column was subjected to a more stringent cleaning procedure. The column was subjected to the following series of cleaning reagents: 0.5 M NaOH 1 M NaCl, 0.1 M acetic acid, 0.1 M acetic acid 1 M NaCl and 70% ethanol. None of the conditions tested were able to remove the dark band from the column (NEG:001:225-245). The colored band was identified as aurintricarboxylic acid, a component present in virgin C5467

medium. A 6 mg/L solution (media concentration) of aurintricarboxylic acid buffered to pH 8.0 with TRIS was loaded directly onto a Q-Sepharose FF column equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0. The colored band appeared at the top of the column immediately following loading. The column was subjected to the same gradient elution and cleaning procedure described above. The aurintricarboxylic acid remained bound to the anion exchange column throughout the run (NEG:001:276-290). The aurintricarboxylic acid readily polymerizes in aqueous solutions resulting in a macromolecule with a high charge density at pH 8.0. The negatively charged macromolecule apparently binds irreversibly to the anion exchange column much like DNA. Repeated applications of this material to the column are likely to decrease the loading capacity, and therefore will, negatively impact the overall column lifetime.

The second medium component was found to co-elute with the PAM. Unconditioned or virgin medium was loaded directly onto the Q-Sepharose FF column that was equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0. The column was again subjected to the same gradient elution conditions described above. A large UV peak in the region where the enzyme typically elutes was observed (NEG:001:248-257). SDS-PAGE analysis of the peak purified from conditioned medium revealed that the material was either low molecular weight proteins or a non-proteinaceous medium component (NEG:001:225-245). The C5467 media contains protein hydrolysates that may account for the large UV peak that is coincident with the enzyme peak. Both medium components, the aurintricarboxylic acid and the unidentified UV peak, are effectively removed prior to chromatography by TFF. The enzyme is retained (retentate) by the 30 kDa membrane and the low molecular weight media components pass through the filter (permeate).

Conditioned medium was concentrated 10-fold and diafiltered using a Pellicon XL device fitted with a regenerated cellulose PLCTK 30 kDa membrane (Millipore). The material was diafiltered with 4 – 5 volumes of 25 mM TRIS, 0.0005% TX-100 pH 7.0 to a final conductivity of approximately 3 mS. The material was loaded onto a Q-Sepharose FF column equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0. The

column was subjected to gradient elution as described above. No discoloration of the resin was observed and the large UV peak that co-eluted with the enzyme was absent. The % recovery and specific activity of the resultant enzyme peak were determined to be 47% and 2.1×10^6 U/mg, respectively (NEG:004:263-283).

Enzyme stability at various stages of the TFF step was investigated. Conditioned medium was initially diafiltered with 50 mM TRIS, 0.001% TX-100 pH 7.0 followed by a 5-fold concentration. Samples of the diafiltration and concentration outputs were analyzed for loss of activity and degradation. No degradation was observed by SDS-PAGE and approximately 95% of the enzyme activity was recovered (NEG:008:245-251).

Anion Exchange Chromatography (Q-Sepharose FF)

Step Description: The anion exchange (AEX) chromatography step provides a gross purification of the PAM from the conditioned CHO cell media. The step mainly removes higher molecular weight proteins. However, some low molecular weight proteins are also removed, including truncated forms of the enzyme. The chromatography step routinely affords a 2 to 3-fold purification of the enzyme. The % recovery of active enzyme is typically 50-75%. DNA that may be present in the feed stream following fermentation of the CHO cells is effectively removed at this stage of the process.

Research and Development: Prior to development of the Q-Sepharose FF step, we briefly investigated purification of PAM using cation exchange (CEX) chromatography. The use of CEX chromatography was investigated with and without the initial TFF step. Purification was carried out on a SP550C (TosoHaas) column employing mobile phases at pH 5.0 - 6.0. In each case, poor resolution and recovery resulted; much of the enzyme activity was identified in the flow through following loading (NEG:004:006-029 and NEG:004:102-119). Decreasing the pH of the mobile phases to facilitate binding was not considered due to the potential lack of stability of the enzyme at low pH. At this point, CEX chromatography was abandoned.

A series of AEX chromatography runs employing gradient elution was investigated on a Q-Sepharose FF column. All Q-Sepharose FF purifications were carried out on a (1.1 x

13.7 cm) column operated at 180 cm/hr. Early in the purification development, the availability of fresh conditioned medium was limited; therefore many of these experiments were performed with freeze/thaw material. Conditioned medium was concentrated/diafiltered by TFF and applied to the column equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0. The column was subjected to a linear salt gradient from 100% A (50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0) to 68% B (50 mM TRIS, 475 mM NaCl, 0.001% TX-100 pH 8.0) over 60 minutes. Similar gradient runs employing HEPES buffers at either pH 7.0 or pH 8.5 were also investigated. Generally, the gradient runs afforded little purification and resulted in poor enzyme specific activity (NEG:004-139-156, NEG:005:119-132, NEG:009:009-022 and NEG:009:024-038). In many instances the enzyme activity was spread out throughout the gradient, perhaps as a result of differential glycosylation.

As the development continued, step elution methods using sequential salt steps were investigated on the Q-Sepharose FF column. Concentrated/diafiltered conditioned media was loaded onto a Q-Sepharose column equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0. The column was subjected to sequential step elution with 175 mM NaCl, 225 mM NaCl and 275 mM NaCl. The enzyme activity was distributed equally between the 175 mM and 225 mM salt steps, with an overall % recovery of approximately 41%. Analysis by SDS-PAGE revealed the presence of 75 kDa enzyme in both the 175 mM and the 225 mM NaCl fractions (NEG:005:198-211). Enzyme activity in both salt steps may be due to differences in glycosylation or perhaps truncated forms of the enzyme that retain activity.

Fresh conditioned medium from either spinner flasks or 10 L bioreactor runs was used for the remainder of the anion exchange experiments. Conditioned CHO medium from spinner flask 00020S3 was subjected to TFF and then loaded onto the Q-Sepharose FF equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0. The enzyme was eluted from the column with a single salt step, 50 mM TRIS, 225 mM NaCl, 0.001% TX-100 pH 8.0. The % recovery and specific activity values of the collected peak were 34% and 1.9×10^6 U/mg, respectively (NEG:008:021-033). Similarly, conditioned CHO

medium from a 10 L bioreactor (00021BR, day 14) was subjected to TFF and purification on the Q-Sepharose using a single 225 mM NaCl step. The % recovery and specific activity values for this run were 37% and 2.3×10^6 U/mg, respectively (NEG:008:065-075).

Step elution using HEPES mobile phases was also investigated in an effort to improve the recovery of active enzyme. Duplicate purification runs employing 50 mM HEPES, 225 mM NaCl, 0.001% TX-100 pH 8.0 as the elution buffer were performed. Conditioned CHO media from a 10 L bioreactor run (Boonton Pilot Facility, run No. 1 day 15) was used as the input material for these purification runs. The % recovery and specific activity for both purification runs were approximately 60% and 3.0×10^6 U/mg, respectively (NEG:008:263-288). The use of HEPES mobile phases apparently improved the % recovery and purity of the enzyme.

The effect of cell viability on the Q-Sepharose FF purification was investigated. Purification of conditioned CHO medium from a 10 L bioreactor run (B00101) harvested on day 14 and day 21 were compared. The cell viability on day 14 and day 21 was measured and determined to be 95% and 48%, respectively. Conditioned CHO medium from day 14 and day 21 were subjected to TFF and Q-Sepharose FF purification using 50 mM HEPES, 225 mM NaCl, 0.001% TX-100 pH 8.0 as the step elution buffer. Purification of the day 14 material resulted in a recovery of 49% and a specific activity of 2.15×10^6 U/mg, while the day 21 run afforded 40% recovery and 2.1×10^6 U/mg (NEG:009:114-125 and NEG:009:149-166). Purifications were also carried out using conditioned CHO medium from a 10 L bioreactor run (00107BR) on day 12 and day 15. The cell viability on day 12 and day 15 were measured and determined to be 86% and 80%, respectively. In each case, the enzyme was eluted from the Q-Sepharose FF column with 50 mM TRIS, 225 mM NaCl, 0.001% TX-100 pH 8.0. The day 12 material resulted in a 62% recovery and a specific activity of 1.9×10^6 U/mg, while the day 15 material gave a 54% recovery and a specific activity of 4.3×10^6 U/mg (NEG:009:195-203 and NEG:009:227-236). Cell viability did not appear to have a significant affect on the overall % recovery or specific activity of the enzyme following AEX

chromatography.

Critical parameters for the Q-Sepharose FF chromatography were identified and investigated to establish working ranges. Scenarios designed to challenge the purification procedure were defined and investigated to determine the impact on the recovery and purity of the enzyme. Two Q-Sepharose FF purification runs were compared using different step elution buffers, either 50 mM TRIS, 250 mM NaCl, 0.001% TX-100 pH 7.8 or 50 mM TRIS, 200 mM NaCl, 0.001% TX-100 pH 8.2 (low pH/high salt and high pH/low salt). The rationale for these experiments was to subject the chromatography to extreme elution conditions in order to define the working ranges. TFF treated conditioned CHO medium from a 10 L bioreactor run (2132-D-1004) was used as the input for purifications. In the low pH/high salt scenario, the conditioned media was loaded onto the column equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 7.8. The enzyme was eluted from the column with 50 mM TRIS, 250 mM NaCl, 0.001% TX-100 pH 7.8. The % recovery and specific activity values of the enzyme were 66% and 2.0×10^6 U/mg, respectively (NEG:010:151-161). The conditioned medium in the high pH/low salt scenario was loaded onto the column equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.2 and eluted with 50 mM TRIS, 200 mM NaCl, 0.001% TX-100 pH 8.2. The % recovery and specific activity of the enzyme in this case were 66% and 2.4×10^6 U/mg, respectively (NEG:010:140-150). Based on these data, the Q-Sepharose FF column can be operated at a pH range of [7.8 – 8.2] and the concentration of sodium chloride in the elution buffer can vary between [200 mM – 250 mM NaCl].

A working capacity for the Q-Sepharose FF column was established to assist in the purification scale-up for 30 L bioreactor runs. The working capacity of the column was defined in two ways: [units of enzyme/mL of resin] and [mg of protein/mL of resin]. Breakthrough for this experiment was defined as a total of $\geq 10\%$ of the enzyme activity identified in the flow through and wash steps. Conditioned CHO medium (100 mL) that had been subjected to TFF (2132-D-1002) was loaded onto a Q-Sepharose FF column equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0. The enzyme

activity and protein concentration of the conditioned medium was determined to be 1.97×10^6 U/mL and 2.41 mg/mL, respectively. The AEX column (Millipore Vantage-L, 1.1 x 13.7 cm) was operated at a linear velocity of 180 cm/hr. Step elution was carried out with 50 mM TRIS, 225 mM NaCl, 0.001% TX-100 pH 8.0. Less than 1% of the total enzyme activity was identified in the column flow through and wash steps. Approximately 51% of the enzyme units were recovered in the main peak. Based on a column volume of 13.0 mL, the working capacity was calculated to be either [1.51×10^7 U/mL of resin] or [18.5 mg of protein/mL resin] (NEG:010:081-092). Future experiments are planned to determine a true loading capacity of the Q-Sepharose FF step.

Hydrophobic Interaction Chromatography (Phenyl-Sepharose FF, low substitution)

Step Description: The Hydrophobic Interaction Chromatography (HIC) step removes the majority of the protein impurities remaining after the AEX step and provides an additional 2-fold purification. SDS-PAGE analyses following HIC revealed a single major band with a few minor lower molecular weight impurities. Typically, the % recovery of active enzyme following HIC is 50 – 75%.

Research and Development: The purification process developed for PAM produced from the B3/A1-7 clone also utilized HIC. Mobile phases containing ammonium sulfate were employed to promote ligand-protein interaction. Prolonged exposure of the enzyme to buffers containing ammonium sulfate resulted in unpredictable precipitation and enzyme denaturation. Other salts in the Hofmeister series, such as sodium chloride and sodium citrate, were investigated in an attempt to minimize these problems.

Reverse linear gradients utilizing mobile phases containing sodium chloride were initially investigated. The input materials for these chromatography studies were either TFF 1 outputs or Q-Sepharose FF outputs. Direct HIC following the initial TFF step was investigated in an attempt to eliminate the AEX step. Typically, the enzyme was diluted with an equal volume of water followed by an equal volume of 20 mM TRIS, 4 M NaCl pH 7.0. Dilution with water prior to salt addition decreases the protein concentration minimizing the possibility of precipitation caused by “salting-out.” The diluted material

was loaded onto a Phenyl-Sepharose FF column equilibrated with 10 mM TRIS, 2.0 M NaCl pH 7.0. The enzyme was eluted from the column with a reverse linear gradient from 100% A (10 mM TRIS, 2.0 M NaCl pH 7.0) to 100% B (10 mM TRIS, pH 7.0) over 68 minutes. Generally, the % recovery of active enzyme was approximately 50% (NEG:005:170-185 and NEG:005:212-226).

Step elution methods employing the sodium chloride mobile phases were also investigated. Enzyme subjected to either TFF 1 or Q-Sepharose FF chromatography was diluted with an equal volume of water followed by an equal volume of 20 mM TRIS, 4 M NaCl pH 7.0. The diluted material was loaded onto an HIC column equilibrated with 10 mM TRIS, 2 M NaCl pH 7.0. The column was washed with additional equilibration buffer followed by a wash with 10 mM TRIS, 1.0 M NaCl pH 7.0. The enzyme was eluted from the column with 10 mM TRIS, 400 mM NaCl pH 7.0. The yield of active enzyme was fair but only marginal purification was achieved (NEG:008:034-046 and NEG:008:135-148). The use of sodium chloride did not appear to cause "salting out" as was the case with ammonium sulfate, however the enzyme was denatured over time in high concentrations of sodium chloride.

Sodium citrate was found to promote binding of the enzyme to the HIC column at relatively low concentrations. Conditioned CHO media subjected to TFF was diluted with an equal volume of water followed by an equal volume of 20 mM TRIS, 1.0 M sodium citrate pH 7.0 and loaded onto a Phenyl-Sepharose FF column equilibrated with 10 mM TRIS, 0.5 M sodium citrate pH 7.0. The column was washed with additional equilibration buffer and stripped with 10 mM TRIS, pH 7.0. No enzyme activity was identified in either the column flow through or wash fractions. Approximately 50% of the enzyme activity was identified in the 10 mM TRIS pH 7.0 fraction (NEG:005:064-076). This experiment was repeated using a lower final concentration of sodium citrate. At the lower salt concentration, the enzyme was diluted with an equal volume of water followed by an equal volume of 20 mM TRIS, 0.6 M sodium citrate pH 7.0 before loading onto the HIC column equilibrated with 10 mM TRIS, 300 mM sodium citrate pH 7.0. The column was washed with additional equilibration buffer and stripped with 10

mM TRIS pH 7.0. Results were essentially identical to the previous run; no activity was identified in either the flow through or wash fractions (NEG:008:203-213).

Gradient elution using the sodium citrate buffer system was investigated to assist in the development of a step elution method. A TFF output was diluted with an equal volume of water followed by an equal volume of 20 mM TRIS, 0.6 M sodium citrate pH 7.0 before loading onto the column equilibrated in 10 mM TRIS, 300 mM sodium citrate pH 7.0. The column was subjected to a reverse linear gradient from 100% A (10 mM TRIS, 300 mM sodium citrate pH 7.0) to 100% B (10 mM TRIS pH 7.0) over 70 minutes. A peak of enzyme activity was identified near the end of the gradient. Other protein impurities eluted in this area of the gradient as well and extended into the 10 mM TRIS pH 7.0 column strip (NEG:008:252-262). Step elution was examined to achieve better separation between the enzyme and the proteins that eluted in the 10 mM TRIS pH 7.0 strip.

The peak fraction (225 mM NaCl) from a Q-Sepharose FF purification (10 L bioreactor run, 00107BR, day 12) was diluted with an equal volume of water followed by an equal volume of 50 mM TRIS, 600 mM sodium citrate pH 7.0. The diluted material was loaded onto a Phenyl-Sepharose column equilibrated with 25 mM TRIS, 300 mM sodium citrate pH 7.0. The column was washed with additional equilibration buffer, followed by a second wash with 25 mM TRIS, 75 mM sodium citrate pH 7.0 and stripped with 25 mM TRIS pH 7.0. All of the enzyme activity was identified in the 75 mM sodium citrate fraction. The % recovery and specific activity were calculated to be 42% and 2.0×10^6 U/mg, respectively (NEG:009:204-218). Better resolution between the enzyme and other protein impurities was achieved using this method.

HIC directly following TFF (no AEX step) was abandoned due to the ineffectiveness of the step to clear DNA from the crude feed stream. Four HIC purifications were performed using the sodium chloride step elution method detailed above. The input for these runs was B3/A1-7 conditioned CHO medium that had been subjected to TFF prior to chromatography. Our internal DNA specification at the time was <10 ng/mg protein. In each case, the level of residual DNA present in the main enzyme peak following HIC

was found to be 6 – 10 times higher than the specification, thereby making the single step HIC purification unacceptable (NEG:006:137-159, NEG:006:176-187 and NEG:006:192-200).

A working capacity for the HIC purification step was investigated to assist in the 30 L scale-up effort. Breakthrough was defined as the presence of $\geq 10\%$ of the enzyme activity in either the column flow through or the wash fractions. Q-Sepharose output (125 mL, 0.368 mg/mL, 710,563 U/mL) from production run 1330-D-1003 was diluted with an equal volume of water followed by an equal volume of 50 mM TRIS, 0.6 M sodium citrate pH 7.0. The diluted material was loaded onto a Phenyl-Sepharose FF column (Millipore Vantage-L, 1.1 x 15.8 cm) equilibrated with 25 mM TRIS, 300 mM sodium citrate pH 7.0. The column was operated at 180 cm/hr. The column was washed with additional equilibration buffer and the enzyme was eluted with 25 mM TRIS, 75 mM sodium citrate pH 7.0. No enzyme activity was identified in either the flow through or the wash fractions. The main peak contained approximately 75% of the enzyme activity units and the specific activity was calculated to be 2.6×10^6 U/mg. Based on a column volume of 15.0 mL, the working capacity was calculated to be 5.92×10^6 U/mL of resin (NEG:010:111-124). A working capacity was not reported in [mg protein/mL of resin] because the protein load on the column was extremely low following AEX. Additional studies are planned to determine a true loading capacity for the HIC step.

The overall time for the HIC unit operation was shortened dramatically by eliminating the water dilution and increasing the operating flow rate to 240 cm/h from 180 cm/h. Minimizing the exposure time to sodium citrate and the amount of time spent on the resin may ultimately help to reduce the loss of active enzyme. The Q-Sepharose FF output from 1330-D-1003 was diluted with an equal volume of 50 mM TRIS, 0.6 M sodium citrate pH 7.0 and purified using the HIC step elution method described in the paragraph above. The % recovery and specific activity of the main peak (25 mM TRIS, 75 mM sodium citrate pH 7.0) was calculated to be 77% and 2.4×10^6 U/mg, respectively (NEG:010:125-139). No protein precipitation was observed during or after the dilution with citrate, however the material must be diluted slowly to avoid the “salting-out”

phenomenon.

The HIC method was challenged by modifying the salt concentrations of the mobile phases to establish working ranges. The effect on % recovery and enzyme specific activity was investigated by performing two different purification procedures. In the first run, the citrate concentration during loading and elution was decreased while in the second run the citrate concentration during loading was decreased while the citrate concentration in the elution buffer was increased. Q-Sepharose FF output from a 10 L bioreactor run (1330-D-1005) was diluted with an equal volume of 50 mM TRIS, 0.5 M sodium citrate pH 7.0 and loaded onto an HIC column equilibrated with 25 mM TRIS, 250 mM sodium citrate pH 7.0. The column was washed with 25 mM TRIS, 50 mM sodium citrate pH 7.0. Approximately 51% of the enzyme activity was identified in the 50 mM sodium citrate fraction with less than 1% identified in the flow through and the wash fractions. The specific activity of the enzyme in the main peak was calculated to be 1.35×10^6 U/mg (NEG:010:175-186). In the second HIC run, the Q-Sepharose FF output was diluted and loaded as described above, however 25 mM TRIS, 100 mM sodium citrate pH 7.0 was employed as the elution buffer. In this case, 50% of the enzyme activity was identified in the 100 mM citrate fraction and less than 2% was identified in the flow through and wash fractions. The specific activity of the enzyme in the 100 mM citrate fraction was 2.5×10^6 U/mg (NEG:010:163-174). The lower specific activity observed in the first HIC run suggested that decreasing the citrate concentration in the elution buffer caused additional proteins that were normally found in the strip to co-elute with the enzyme, thus decreasing the final purity. In contrast, increasing the citrate concentration in the elution buffer did not appear to decrease purity. Based on these data, the concentration of sodium citrate in the elution buffer can vary between 75 – 100 mM without compromising final purity.

Tangential Flow Filtration No. 2 (TFF 2)

Step Description: TFF 2 is utilized to concentrate and diafilter the Phenyl-Sepharose FF output prior to virus filtration. The HIC output is subjected to TFF immediately to

minimize inactivation of the enzyme by prolonged storage in the elution buffer (25 mM TRIS, 75 mM sodium citrate pH 7.0). The Phenyl-Sepharose FF output is concentrated approximately 3-fold and diafiltered to place the enzyme into a suitable buffer for virus filtration and subsequent storage. The TFF 2 step employs a Pellicon 2 Module fitted with a regenerated cellulose PLCTK 30 kDa membrane (Millipore).

Research and Development: The TFF 2 procedure was originally developed for the PAM produced using the B3/A1-7 clone. The enzyme has been shown to be stable in 50 mM TRIS, 25 mM NaCl, 0.0005% TX-100 pH 8.0 under various conditions, for this reason, no additional R&D was attempted.

Representative Small-Scale Purification of the PAM from C5467 Conditioned CHO Media

A sample of conditioned CHO cell media (500 mL) from a 10 L bioreactor run (00107BR, day 15) was concentrated 8-fold and diafiltered against 50 mM TRIS, 0.001% TX-100 pH 8.0. Approximately one-third of the TFF 1 output was purified using a scaled-down version of the purification process described above (NEG:009:219-249). The purification data are summarized in Table 8. The step yields for this purification run were relatively poor and the specific activity of the enzyme actually decreased following the HIC step. However, the SDS-PAGE analysis revealed that overall, α -amidating enzyme was purified to a single major band at approximately 75 kDa (Figure 12, lane 10). The gel and densitometry scan (Figure 13) clearly indicated that the enzyme is highly pure (approximate purity, 67%) with only minor low molecular weight impurities present. The enzyme was inactivated over time in many of the purification buffers; therefore the execution of the process must proceed without delay between steps. The variability in the purification process was attributed to the presence of inactive enzyme, which decreases the overall yield and specific activity. More importantly, both the purification and fermentation development preceded concurrently, which may also account for some of the variability observed. Step yields and specific activity at the production scale have subsequently been found to be much better, perhaps as a result of moving the material through the process faster and assaying the fractions promptly.

Table 8 Small-Scale Purification Of PAM From UGL 73-26/M Conditioned CHO Media

Process Step	Yield (U)	Step Yield (%)	Overall Yield (%)	Total Protein (mg)	Specific Activity (U/mg)
TFF #1	5.89E+07	NA	NA	36.16	1.63E+06
AEX	3.17E+07	53.7	53.7	7.42	4.27E+06
HIC	1.09E+07	34.5	18.5	4.25	2.57E+06
TFF #2	8.18E+06	74.8	13.9	2.41	3.39E+06

Summary of PAM Downstream Purification Process

Purification procedures for each process step that reflect the information that was forwarded to the pilot facility in the technology transfer are detailed below. Data from a representative small-scale purification run, including the SDS-PAGE and densitometry scan, are included in Table 8 and Figures 11 and 12.

TFF 1 Procedure: The clarified conditioned CHO cell media is concentrated 5-fold and diafiltered with 50 mM TRIS, 0.001% TX-100 pH 8.0 until a final conductivity of 4 – 6 mS is achieved. The transmembrane pressure is maintained at ≤ 10 psi throughout the filtration.

AEX Procedure: The clarified conditioned CHO cell media that has been concentrated/diafiltered by TFF is applied to a Q-Sepharose FF (Amersham Biosciences) column equilibrated with 50 mM TRIS, 120 mM NaCl, 0.001% TX-100 pH 8.0. The column is operated at 180 cm/hr and the UV absorbance is monitored at 280 nm. The column is washed with additional equilibration buffer and the PAM is eluted from the column with 50 mM TRIS, 225 mM NaCl pH 8.0. The column is cleaned with 2.0 M NaCl and sanitized with 1.0 M NaOH. The column is stored between runs in 10 mM NaOH.

HIC Procedure: The Q-Sepharose output is diluted with an equal volume of 50 mM TRIS, 600 mM citrate pH 7.0 and loaded onto a Phenyl-Sepharose FF (Amersham Biosciences) equilibrated with 25 mM TRIS, 300 mM citrate pH 7.0. The column is

operated at 240 cm/hr and the UV absorbance is monitored at 280 nm. The column is washed with 25 mM TRIS, 300 mM citrate pH 7.0 and the PAM is eluted from the column with 25 mM TRIS, 75 mM citrate pH 7.0. The column is cleaned and sanitized with 25 mM TRIS pH 7.0 and 1.0 M NaOH, respectively. The column is stored between runs in 10 mM NaOH.

TFF 2 Procedure: The Phenyl-Sepharose FF output is initially concentrated approximately 3-fold, diafiltered with 50 mM TRIS, 25 mM NaCl, 0.0005% TX-100 pH 8.0 and concentrated further to a suitable volume for virus filtration and storage.

10 L Stirred Tank Bioreactor Process Runs

Overview of the 10 L Fermentation and Purification Process for the α -Amidating Enzyme

Purified α -AE was prepared following several 10 L stirred tank bioreactor runs. The process steps to derive the α -amidating enzyme were a 14 day inoculum phase, a 17 day fermentation phase and a 2 day purification scheme, detailed above and in Figure 14. The culture media used in these experiments was a protein-free CHO media, C5467 (Sigma-Aldrich). All process details from these runs can be found in the appropriate batch records.

The process flow diagram, Figure 14, illustrates the final process steps developed for α -AE /PAM produced using the UGL 73-26/M clone.

Phase 1 – Inoculum

To prepare an inoculum for a 10 L stirred tank bioreactor a single vial of the UGL 73-26/M MWCB00 was thawed. The cells from the cryovial were removed and placed into fresh media. The cell pellet was resuspended into new media and added to a spinner flask containing C5467 CHO media. During the next 14 days the culture was expanded into four flasks containing 400 mL media plus cells (see Inoculum Scheme, Fig. 15).

The culture was tested for α -AE expression at the end of the inoculum phase¹². On day 12, the average enzyme activity concentration was >13,000 U/mL, see Table 9 panel C. A cell count of the culture and cell viability was done each time the inoculum culture was modified. The culture viability was generally greater than 95% during this phase, see Table 9 panel A. The inoculum culture average total viable cells count was 2.03×10^9 on day 14, see Table 9 panel B.

Table 9 Cell Culture Statistics – Inoculum Phase

Panel A

Percent Cell Viability (%)							
Day of Inoculum	2130-1003	2130-1004	2130-1005	2130-1006	2130-1009	2130-1010	Overall
3	98.0	97.5	100.0	98.0	98.5	98.5	
5	98.5	97.5	98.5	98.0	100.0	96.5	
7	97.5	97.5	97.0	98.5	99.0	99.0	
10A	98.0	99.0	98.5	97.0	99.0	98.0	
10B	98.0	98.0	97.5	98.5	99.0	98.5	
12A	98.5	100.0	98.5	99.0	97.5	99.0	
12B	98.5	100.0	99.0	100.0	98.0	99.0	
12C	97.5	100.0	97.0	98.5	98.5	97.5	
12D	98.0	100.0	97.5	99.5	99.0	98.5	
14A	97.5	100.0	93.5	97.5	100.0	95.0	
14B	96.5	100.0	97.5	99.5	98.0	96.0	
14C	98.0	100.0	99.0	98.0	99.0	97.0	
14D	96.0	100.0	98.5	97.5	98.0	97.0	
Day 14 Average	97.0	100.0	97.1	98.1	98.8	96.3	97.9
SD							1.7

Panel B

Total Number of Viable Cells ($\times 10^7$)							
Day of Inoculum	2130-1003	2130-1004	2130-1005	2130-1006	2130-1009	2130-1010	Overall
3	3.6	1.5	1.3	2.2	1.6	2.4	
5	14.7	6.3	5.7	6.1	5.5	6.3	
7	28.3	20.6	12.5	22.0	15.4	25.4	
10A	34.0	38.0	29.8	38.0	32.0	40.0	
10B	32.0	38.0	35.6	37.0	33.0	33.0	
12A	34.0	40.0	34.0	40.0	33.0	40.0	
12B	29.7	44.0	32.0	41.0	32.0	34.0	
12C	24.0	48.0	31.0	37.0	28.0	30.0	
12D	25.8	48.0	30.0	37.0	28.0	30.0	
14A	38.0	67.0	56.0	48.0	44.0	45.0	
14B	37.0	78.0	58.0	52.0	55.0	43.0	
14C	39.0	44.0	53.0	58.0	42.0	40.0	
14D	30.0	70.0	72.0	60.0	49.0	40.0	
Day 14 Total	144.0	259.0	239.0	218.0	190.0	168.0	203.0
SD							43.7

Panel C

Enzyme Activity (U/mL)							
Day of Inoculum	2130-1003	2130-1004	2130-1005	2130-1006	2130-1009	2130-1010	Overall
12A	13,112	15,630	12,301	14,408	10,093	11,951	
12B	13,398	16,532	14,478	14,380	9,363	10,201	
12C	13,456	16,224	13,569	15,450	9,563	11,306	
12D	12,905	16,057	15,568	13,014	9,490	12,183	
Day 12 Average	13,218	16,111	13,979	14,313	9,627	11,410	13,110
SD							2,255

Phase 2 - Fermentation

A 10 L bioreactor was initiated at the completion of each inoculum phase. The bioreactor was seeded at 1×10^5 cells/mL in virgin protein-free media with L-glutamine (Sigma, C5467). The bioreactor parameters were set at the following set points; temperature = 37°C, RPM = 60, pH = no pH set point maintained, the pH was allowed to drift (at the onset, the pH is not allowed to be greater than pH 7.5) and the DO set point is 70% DO. The dissolved oxygen concentration of the virgin media was greater than 70%, the dissolved oxygen concentration of the bioreactor was allowed to drift to 70% and then maintained at that set point. The bioreactor was supplemented with 2 g/L glucose on days 5, 10 and 14. The conditioned media was harvested on day 17. The harvest material was clarified through a Millipore Opticap Filter. The first two fermentations described below were harvested on day 18 instead of day 17, 2131-D-1003 and 2131-D-1004. The maximum cell density of the culture was reached and maintained between days 10 and 17/18. The average maximum cell density from these fermentations was $1.5\text{--}1.6 \times 10^6$ cells/mL, Table 10 panel B. The viability of the culture was >80% for the initial 14 days of culture and the average viability on the day of harvest was 76.0%, Table 10 panel A. The productivity of the culture was evaluated at harvest, day 17/18. The average clarified harvest contained 378,567 units α -AE/mL, Table 10 panel C.

Table 10 Cell Culture Statistics – Fermentation Phase

Panel A

Percent Cell Viability (%)								
Day of Fermentation	2131-1003	2131-1004	2131-1005	2131-1006	2131-1009	2131-1010	Average	SD
3	97.5	99.0	96.0	100.0	96.5	97.5	97.8	1.5
5	98.5	99.0	99.0	98.5	98.5	98.5	98.7	0.3
10	95.0	95.5	92.5	91.0	94.0	93.0	93.5	1.7
14	93.5	86.5	82.5	83.5	86.0	91.0	87.2	4.3
17/18	73.5	72.5	75.0	74.5	78.5	82.0	76.0	3.6

Panel B

Viable Cells Density (cells/mL x 10 ⁶)								
Day of Fermentation	2131-1003	2131-1004	2131-1005	2131-1006	2131-1009	2131-1010	Average	SD
3	0.4	0.3	0.4	0.4	0.3	0.3	0.4	0.1
5	0.6	0.6	0.8	0.7	0.8	0.4	0.7	0.1
10	1.2	1.3	1.9	1.9	1.4	1.5	1.5	0.3
14	0.9	1.1	2.0	1.9	1.9	1.6	1.6	0.5
17/18	1.4	1.3	2.0	1.4	1.9	1.5	1.6	0.3

Panel C

Enzyme Activity (U/mL)								
Day of Fermentation	2131-1003	2131-1004	2131-1005	2131-1006	2131-1009	2131-1010	Average	SD
17/18	412,120	398,397	371,950	505,269	383,975	319,693	378,567	61,174

Phase 3 - Purification

The purification process has 5 distinct steps as detailed above. The clarified harvest was concentrated and diafiltered against 50 mM TRIS, 0.001% TX-100, pH 8.0. The enzyme material was concentrated approximately 4-fold on average (data not shown, see batch records). This process material was applied to a Q-sepharose column and eluted following a NaCl step gradient in a 50 mM TRIS, 0.001% Triton buffer containing 225 mM NaCl. The enzyme solution was diluted with 50 mM TRIS, 600 mM Citrate, pH 7.0 and applied to a phenyl sepharose column. The α -amidating enzyme was eluted from the column following a citrate step gradient. The enzyme material was concentrated to a final volume of approximately 1 L. Four of the six batches of enzyme were processed through a virus removal filter. Two batches were processed through a Millipore NFP filter (1330-D-1005 and 1330-D-1006) and two batches through a Pall Trincor DV50 filter (1330-1009 and 1330-1010). Data from each of the process steps are shown in Table 11 below.

The first step, TFF 1, was a nearly quantitative step with regard to α -amidating enzyme activity units retention. The TFF concentrate from a 10 L bioreactor run has a mean specific activity of 1.60×10^6 units/mg protein, Table 11. The TFF 1 concentrate having been applied to a Q-sepharose chromatography column was eluted from the column in approximately 3 L. The specific activity of the enzyme has increased in this process step approximately 2-fold to 3.33×10^6 units/mg. The protein concentration and enzyme activity concentration of the Q-sepharose eluate were very consistent, see Table 11. Following phenyl-sepharose chromatography the average specific activity of the enzyme increased from 3.33×10^6 to 4.96×10^6 units/mg protein. The phenyl-sepharose eluate was concentrated to 1 L using a second TFF step. There is no change in specific activity following this concentration step, Table 11. The TFF 2 concentrates from some enzyme batches were applied to a virus removal filter. The specific activities for 3 of the 4 batches of enzyme decreased, Table 11. The average specific activity for the final purified enzyme decreased 350,000 units/mg to 4.61×10^6 units/mg protein. The

decrease in specific activity was likely to be due to inactivation of some portion of the enzyme.

Table 11 Individual Purification Process Step

TFF 1

Batch #	Enzyme Activity (U/mL)	Protein (mg/mL)	Specific Activity (U/mg)
1330-D-1003	3,575,083	1.850	1,932,477
1330-D-1004	1,308,056	0.572	2,286,811
1330-D-1005	1,545,462	1.106	1,397,344
1330-D-1006	1,560,833	1.244	1,254,689
1330-1009	1,244,457	0.925	1,345,359
1330-1010	1,533,800	1.481	1,035,652
Mean	1,794,615	1.196	1,542,055
SD	882,523	0.443	470,265
% CV	49.2	37.0	30.5

Q Sepharose FF

Batch #	Enzyme Activity (U/mL)	Protein (mg/mL)	Specific Activity (U/mg)
1330-D-1003	710,563	0.244	2,912,143
1330-D-1004	808,386	0.222	3,641,378
1330-D-1005	778,492	0.255	3,052,910
1330-D-1006	816,303	0.204	4,001,485
1330-1009	778,864	0.243	3,205,202
1330-1010	807,958	0.257	3,143,805
Mean	783,428	0.238	3,326,154
SD	39,140	0.021	412,162
% CV	5.0	8.7	12.4

Table 11 continued

Phenyl Sepharose FF

Batch #	Enzyme Activity (U/mL)	Protein (mg/mL)	Specific Activity (U/mg)
1330-D-1003	274,877	0.093	2,955,667
1330-D-1004	413,767	0.057	7,259,070
1330-D-1005	168,186	0.029	5,799,517
1330-D-1006	296,924	0.080	3,711,550
1330-1009	357,854	0.075	4,771,387
1330-1010	325,502	0.062	5,250,032
Mean	306,185	0.066	4,957,871
SD	83,338	0.022	1,528,801
% CV	27.2	33.7	30.8

TFF 2

Batch #	Enzyme Activity (U/mL)	Protein (mg/mL)	Specific Activity (U/mg)
1330-D-1003	919,409	0.255	3,605,525
1330-D-1004	1,325,542	0.309	4,289,780
1330-D-1005	1,081,522	0.220	4,916,009
1330-D-1006	1,560,058	0.315	4,952,565
1330-1009	2,085,104	0.363	5,744,089
1330-1010	1,423,017	0.355	4,008,499
Mean	1,399,109	0.303	4,586,078
SD	408,277	0.056	770,328
% CV	29.2	18.5	16.8

Table 11 continued

Viral Filtration

Batch #	Enzyme Activity (U/mL)	Protein (mg/mL)	Specific Activity (U/mg)
1330-D-1003			
1330-D-1004			
1330-D-1005	989,040	0.196	5,046,122
1330-D-1006	1,124,322	0.298	3,772,893
1330-1009	1,528,944	0.300	5,096,480
1330-1010	1,577,241	0.349	4,519,315
Mean	1,304,887	0.286	4,608,703
SD	292,541	0.064	615,313
% CV	22.4	22.5	13.4

The data illustrating the consistency of each step of a purification process are important. However, just as important is the percent recovery of the desired product at each step of the process. Table 12 shows the percent recovery for each step of the α -amidating enzyme purification process and the overall yield of the process after four or five of the process steps. Table 12 shows that the enzyme was quantitatively recovered from both of the TFF steps. The average percent recoveries for the chromatography steps, Q-sepharose and phenyl sepharose, are 65.3% and 72.0%, respectively. Both of the virus removal filters tested recovered ~80% of the α -AE activity. The overall yield of the enzyme purification process from clarified harvest material through TFF 2 or viral filtration was 40.8% or 38.8%, respectively.

Table 12 Cell Culture Statistics – Purification Phase

Batch #	Purification Process Step (% Recovery)						Total Recovery Through TFF2	Total Recovery Through DV50 Viral Filtration
	TFF 1	Q- Sephrose	Phenyl- Sephrose	TFF 2	Viral Filtration			
1330-D-1003	193	33	37	138			33	
1330-D-1004	88	87	59	90			41	
1330-D-1005	117	57	76	79	91		39	36
1330-D-1006	69	90	80	77	77		38	29
1330-1009	95	66	90	92	80		52	42
1330-1010	122	59	90	91	114		42	48
Mean	114.0	65.3	72.0	94.5	90.5		40.8	38.8
SD	43.3	21.1	20.6	22.3	16.8		6.3	8.1

Conclusions

A stable well characterized CHO cell line, UGL 73-26/M, that expresses high levels of α -AE activity has been developed. High levels of enzyme expression are achieved in a 17 day batch fermentation process, which utilizes a non-animal source, low protein containing tissue culture medium C5467 (Sigma). Critical fermentation parameters such as pH, DO and glucose concentration have been investigated and optimized. A robust two-step downstream purification process that is capable of purifying the enzyme to near homogeneity has also been developed. The consistency of the fermentation and purification processes are well suited for scale-up to the manufacturing level.

Examples are set forth below showing production of amidated product using PAM expressed by cells of the invention.

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EXAMPLE 1: Conversion Of Glycine-Extended Parathyroid Hormone Fragment To Amidated Counterpart Using Peptidylglycine α -amidating Monooxygenase.

Amidation of rhPTH(1-34)Gly35-OH Using Pyruvate

The components and final concentrations used for amidation of rhPTH(1-34)Gly35-OH are shown in the Table 12. A brief description of the amidation follows.

Table 12	
α-Amidation of rhPTH(1-34)Gly35-OH	
Reagent	Final Concentration
rhPTH(1-34)Gly35-OH	2 mg/mL
250 mM MES pH 6.3	30 mM
3 mM Cupric Sulfate	0.5 μ M
100 mM Sodium Ascorbate	2 mM
Oxygen	The dissolved oxygen concentration is maintained at or near saturation.
400 mM Sodium Pyruvate	8 mM
250 mM Potassium Iodide	5 mM
190 Proof Ethanol	1%
PAM	30,000 U/mL

- Approximately 12.4 grams of rhPTH(1-34)Gly35-OH in 1,900 mL of 25 mM MES, 200 mM NaCl pH 6.0 was charged into a glass vessel fitted with an agitator and gas sparger.
- To this solution, the following components were added in the order listed: 3,025 mL water, 741 mL 250 mM MES pH 6.3, 1.03 mL 3 mM cupric sulfate, 124 mL potassium iodide, 62 mL 190 proof ethanol, 124 mL 400 mM sodium pyruvate and 124 mL 100 mM sodium ascorbate.
- The reaction vessel was placed into a water-bath and the reaction mixture was heated to 25 – 27°C with stirring.
- The pH of the reaction mixture was adjusted by to 5.8 with 21 mL of 2 M HCl. Oxygen sparging was initiated; the sparging rate was adjusted to avoid excessive foaming of the reaction mixture.

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- 47 mL of PAM was added and the reaction mixture was incubated at 25 – 27°C for 4 hours and 35 minutes (oxygen sparging was performed throughout the incubation period).
- The reaction mixture was acidified to pH 2.4 with 74 mL of 2 M HCl.

The PAM enzyme used in this example was expressed from preferred CHO K1 cells of the invention, which were constructed as described *supra*, and are designated herein as UGL 73-26/M. This cell line was also used to provide the ATCC deposit discussed *supra*.

The glycine extended precursor may be obtained from known sources, or may be produced in known ways. For example, it may be produced by fermentation in a manner analogous to that described in U.S. patent 6,103,495 (Examples 1-2 thereof), and purified as described in U.S. Patent 6,103,495 (Example 3 thereof) prior to amidation. The particular precursor used in the present amidation described above was expressed by a cell line of co-pending U.S. Patent Application Serial No. 11/076,260, filed March 9, 2005, and published October 6, 2005 as Publication No. US 2005/0221442.

In instances where the enzyme used for amidation is peptidyl glycine alpha-hydroxylating monooxygenase (PHM), the same reaction mixture may be used as that described above, substituting PHM for PAM. In addition, at the end of the 4 to 6 hour incubation period, the pH of the reaction mixture is increased by the addition of base to between 8 and 9. The reaction mixture is agitated for an additional 4 to 8 hours prior to terminating the reaction. PHM may be obtained by expressing PAM as taught herein, followed by separating the PHM catalytic domain from the rest of the PAM molecule. Alternatively, the vectors used herein may be modified to stop translation after PHM but before the PAL catalytic domain. Alternatively, the vector may be constructed, in the first instance, with a PHM coding region in place of the PAM coding region discussed *supra*. PHM may be obtained by expressing only the N-terminal portion of PAM (about the first 40 kDa). PAMs and the location of their

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catalytic domains are reported in the literature. Any such PAMs or PHMs are believed useful in accordance with the present invention. See e.g. Mizuno et al, BBRC Vol. 148, No. 2, pp. 546-52 (1987), the disclosure of which is incorporated herein by reference. See especially Mizuno's "AE1." Frog skin is known to express PHM naturally.

EXAMPLE 2: Post-Amidation Purification

Cation Exchange (CEX) Chromatography

Purification of rhPTH(1-34)-NH₂ from residual rhPTH(1-34)Gly35-OH was achieved using CEX chromatography. A brief description of the CEX chromatography method is described below. The acidified amidation output was loaded onto a Toyopearl SP650M (Tosoh Bioscience LLC) column, 9 cm x 19 cm, equilibrated with 25 mM MES pH 6.5. The column was operated at 180 cm/hr and the UV absorbance of the column effluent was monitored at 280 nm. The column was washed with 25 mM MES pH 6.5 until the pH of the column effluent pH returned to 6.5. The column was washed with 25 mM MES, 80 mM NaCl pH 6.5 until the wash peak completely eluted and a stable UV baseline was achieved. The product, rhPTH(1-34)-NH₂, was eluted from the column with 25 mM MES, 200 mM NaCl pH 6.5. The entire UV peak was collected; fractions were screened by RP-HPLC to determine pooling criteria.

Reversed-Phase (RP) Chromatography

RP chromatography was utilized to exchange the salt form of the peptide from chloride to acetate; RP chromatography provides marginal purification of the peptide. The CEX chromatography output was diluted with 3 volumes of 333 mM sodium acetate and mixed thoroughly. The mixture was allowed to stand for 75 minutes at room temperature prior to loading. The acetate diluted sample was loaded onto a Amberchrom CG300 M (Tosoh Bioscience LLC) column, 6 cm x 17 cm, equilibrated with 250 mM sodium acetate pH 7.5. The column was operated at 180 cm/hr and the UV absorbance of the column effluent was monitored at 280 nm. The

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column was washed with 250 mM sodium acetate pH 7.5 for 60 minutes. The column was equilibrated in 0.1% acetic acid. The product, rhPTH(1-34)-NH₂, was eluted from the column with 0.1% acetic acid, 40% ethanol. The entire UV peak was collected.

Characterization of rhPTH(1-34)-NH₂

The RP chromatography output was concentrated to a white flocculent powder by lyophilization, yielding 11.8 grams (95% overall yield from amidation) of rhPTH(1-34)-NH₂. The molecular mass for rhPTH(1-34)-NH₂ was determined to be 4,116.9 Da by electrospray ionization mass spectrometry (ESI-MS), which was consistent with the calculated average molecular mass of 4,116.8 Da.

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WHAT IS CLAIMED IS

1. A CHO K1 cell transfected with an expression vector for expressing peptidylglycine alpha-amidating monooxygenase.
2. The transformed cell of claim 1 wherein said expression vector has a coding region with nucleic acids encoding peptidylglycine alpha-amidating monooxygenase operably linked to a control region including a ribosome binding site, a promoter and an SV40 enhancer upstream of said promoter.
3. The transformed cell of claim 1 wherein said expression vector has a coding region with nucleic acids encoding peptidylglycine alpha-amidating monooxygenase operably linked to a control region having a ribosome binding site and, a human metallothionin IIA promoter.
4. The transformed cell of claim 3 wherein said vector further includes an enhancer upstream of said promoter.
5. The transfected cell of claim 4 wherein said enhancer is an SV40 enhancer.
6. A recombinant expression vector having a coding region with nucleic acids encoding peptidylglycine alpha-amidating monooxygenase, operably linked to a control region including a ribosome binding site, a promoter and an SV40 enhancer upstream of said promoter.
7. A recombinant expression vector having a coding region with nucleic acids encoding peptidylglycine alpha-amidating monooxygenase, operably linked to

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a control region including a ribosome binding site and, a human metallothionin IIA promoter.

8. The vector of claim 7 wherein said vector further includes an enhancer upstream of said promoter.

9. The vector of claim 8 wherein said enhancer is an SV40 enhancer.

10. A method of preparing a cell line for the expression of peptidylglycine alpha-amidating monooxygenase, said method comprising the steps of:

- (A) Transfecting potential host cells in the presence of first, second and third expression vectors, wherein said first vector includes a coding region encoding a first selectable marker, wherein said second vector includes a coding region encoding a second selectable marker, wherein said third vector includes a coding region encoding peptidylglycine alpha-amidating monooxygenase, wherein the concentration ratio of said third vector to said first vector is at least 3:1, and wherein the concentration ratio of said third vector to said second vector is at least 3:1;
- (B) Subjecting the cells resulting from step (A) to selectable pressure to select cells that have been transfected with said first vector;
- (C) Subjecting the cells resulting from step (B) to selectable pressure to select cells that have been transfected with said second vector;
- (D) Subjecting the cells resulting from step (C) to limiting dilution and selecting cells expressing peptidylglycine alpha-amidating monooxygenase.

11. The method of claim 10 further comprising the step of amplifying peptidylglycine alpha-amidating monooxygenase by subjecting cells resulting from step (D) to amplifying selective pressure.

12. The method of claim 10 wherein said first selectable marker is a gene encoding neomycin resistance.

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13. The method of claim 10 wherein said second selectable marker is a gene encoding dihydrofolate reductase.
14. The method of claim 11 wherein at least one of said selectable markers is a gene encoding dihydrofolate reductase and wherein said amplification is conducted in the presence of methotrexate.
15. The method of claim 10 wherein the concentration ratio of said third vector to said first vector is at least 10:1 and the concentration ratio of said third vector to said second vector is at least 10:1.
16. The method of claim 10 wherein said third vector includes a coding region having nucleic acids encoding peptidylglycine alpha-amidating monooxygenase, operably linked to a control region including a ribosome binding site, a promoter and an SV40 enhancer upstream of said promoter.
17. The method of claim 10 wherein said third vector includes a coding region having nucleic acids encoding peptidylglycine alpha-amidating monooxygenase, operably linked to a control region including a ribosome binding site, human metallothionin to a promoter and an enhancer upstream of said promoter.
18. The method of claim 17 wherein said enhancer is an SV40 enhancer.
19. The method of claim 10 wherein said potential host cells include CHO K1 cells.
20. A cell line that results from the method of claim 10.
21. A cell line comprising host cells transfected with the vector of claim 6.

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22. Peptidylglycine alpha-amidating monooxygenase expressed by the cells or cell lines of any of claims 1- 5, 20 or 21.
23. A method for the *in vitro* production of an amidated product, said method comprising reacting a precursor having a glycine residue, in free acid form and attached to a carbonyl group, in the presence of the peptidylglycine alpha-amidating monooxygenase of claim 22.
24. The method of claim 23 wherein said precursor is a peptide with a C-terminal glycine.
25. An amidated product resulting from the method of claim 23.
26. Cell line UGL 73-26/M.
27. Peptidylglycine alpha-amidating monooxygenase expressed by cell line UGL 73-26/M.
28. The method of claim 23 wherein said peptidylglycine alpha-amidating monooxygenase was expressed by cell line UGL 73-26/M.
29. Amidated product resulting from the method of claim 28.
30. A method of purifying peptidylglycine alpha-amidating monooxygenase following expression and secretion into culture media, wherein said method includes the steps of :
- (A) Subjecting an impure sample of peptidylglycine alpha-amidating monooxygenase to anion exchange chromatography wherein elution is isocratic;
 - (B) Subjecting the eluant of step (A) to hydrophobic interaction chromatography, wherein ammonium sulfate is not used and wherein elution is isocratic.

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31. The method of claim 30 wherein sodium citrate is utilized to facilitate column binding in step (B).

32. A method for the *in vitro* production of an amidated product said method comprising:

(A) forming a hydroxylated intermediate by reacting a precursor having a glycine residue, in free acid form and attached to a carbonyl group, in the presence of peptidylglycine alpha-hydroxylating monooxygenase; and

(B) simultaneously or subsequently reacting said intermediate with either a Lewis base or peptidyl alpha-hydroxyglycine alpha-amidating lyase;

wherein said peptidylglycine alpha-hydroxylating monooxygenase has been derived by separating the peptidylglycine alpha-hydroxylating monooxygenase catalytic domain from peptidylglycine alpha-amidating monooxygenase expressed by the cells or cell lines of claims 1-5, 20 or 21.

33. The method of claim 32 wherein a Lewis base is utilized for step (B).

34. A CHO K1 cell transfected with an expression vector for expressing peptidylglycine alpha-hydroxylating monooxygenase.

35. The transformed cell of claim 34 wherein said expression vector has a coding region with nucleic acids encoding peptidylglycine alpha- hydroxylating monooxygenase operably linked to a control region including a ribosome binding site, a promoter and an SV40 enhancer upstream of said promoter.

36. The transformed cell of claim 34 wherein said expression vector has a coding region with nucleic acids encoding peptidylglycine alpha- hydroxylating monooxygenase operably linked to a control region having a ribosome binding site and, a human metallothionin IIA promoter.

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37. The transformed cell of claim 36 wherein said vector further includes an enhancer upstream of said promoter.
38. The transformed cell of claim 37 wherein said enhancer is an SV40 enhancer.
39. A recombinant expression vector having a coding region with nucleic acids encoding peptidylglycine alpha- hydroxylating monooxygenase, operably linked to a control region including a ribosome binding site, a promoter and an SV40 enhancer upstream of said promoter.
40. A recombinant expression vector having a coding region with nucleic acids encoding peptidylglycine alpha-hydroxylating monooxygenase, operably linked to a control region including a ribosome binding site and, a human metallothionin IIA promoter.
41. The vector of claim 40 wherein said vector further includes an enhancer upstream of said promoter.
42. The vector of claim 41 wherein said enhancer is an SV40 enhancer.
43. A method of preparing a cell line for the expression of peptidylglycine alpha- hydroxylating monooxygenase, said method comprising the steps of:
(A) Transfecting potential host cells in the presence of first, second and third expression vectors, wherein said first vector includes a coding region encoding a first selectable marker, wherein said second vector includes a coding region encoding a second selectable marker, wherein said third vector includes a coding region encoding peptidylglycine alpha-hydroxylating monooxygenase, wherein the concentration ratio of said third vector to said first vector is at least 3:1, and wherein the concentration ratio of said third vector to said second vector is at least 3:1;

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- (B) Subjecting the cells resulting from step (A) to selectable pressure to select cells that have been transfected with said first vector;
- (C) Subjecting the cells resulting from step (B) to selectable pressure to select cells that have been transfected with said second vector;
- (D) Subjecting the cells resulting from step (C) to limiting dilution and selecting cells expressing peptidylglycine alpha- hydroxylating monooxygenase.

- 44. The method of claim 43 further comprising the step of amplifying peptidylglycine alpha- hydroxylating monooxygenase by subjecting cells resulting from step (D) to amplifying selective pressure.
- 45. The method of claim 43 wherein said first selectable marker is a gene encoding neomycin resistance.
- 46. The method of claim 43 wherein said second selectable marker is a gene encoding dihydrofolate reductase.
- 47. The method of claim 44 wherein at least one of said selectable markers is a gene encoding dihydrofolate reductase and wherein said amplification is conducted in the presence of methotrexate.
- 48. The method of claims 43 wherein the concentration ratio of said third vector to said first vector is at least 10:1 and the concentration ratio of said third vector to said second vector is at least 10:1.
- 49. The method of claim 43 wherein said third vector includes a coding region having nucleic acids encoding peptidylglycine alpha-hydroxylating monooxygenase, operably linked to a control region including a ribosome binding site, a promoter and an SV40 enhancer upstream of said promoter.

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50. The method of claim 43 wherein said third vector includes a coding region having nucleic acids encoding peptidylglycine alpha-hydroxylating monooxygenase, operably linked to a control region including a ribosome binding site, human metallothionin to a promoter and an enhancer upstream of said promoter.

51. The method of claim 50 wherein said enhancer is an SV40 enhancer.

52. The method of claim 43 wherein said potential host cells include CHO K1 cells.

53. A cell line that results from any of the methods of claims 43-52.

54. A cell line comprising host cells transfected with the vector of any of claims 39-42.

55. Peptidylglycine alpha-hydroxylating monooxygenase expressed by the cells or cell lines of any of claims 34-38.

56. A method for the *in vitro* production of an amidated product, said method comprising reacting a precursor having a glycine residue, in free acid form and attached to a carbonyl group, in the presence of the peptidylglycine alpha-hydroxylating monooxygenase of claim 55, followed by further reaction in the presence of Lewis base or peptidyl alpha-hydroxyglycine alpha-amidating lyase.

57. The method of claim 56 wherein said precursor is a peptide with a C-terminal glycine.

58. An amidated product resulting from the method of claim 56.

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59. A method of purifying peptidylglycine alpha- hydroxylating monooxygenase following expression and secretion into culture media, wherein said method includes the steps of :

- (A) Subjecting an impure sample of peptidylglycine alpha- hydroxylating monooxygenase to anion exchange chromatography wherein elution is isocratic;
- (B) Subjecting the eluant of step (A) to hydrophobic interaction chromatography, wherein ammonium sulfate is not used and wherein elution is isocratic.

60. The method of claim 59 wherein sodium citrate is utilized to facilitate column binding in step (B).

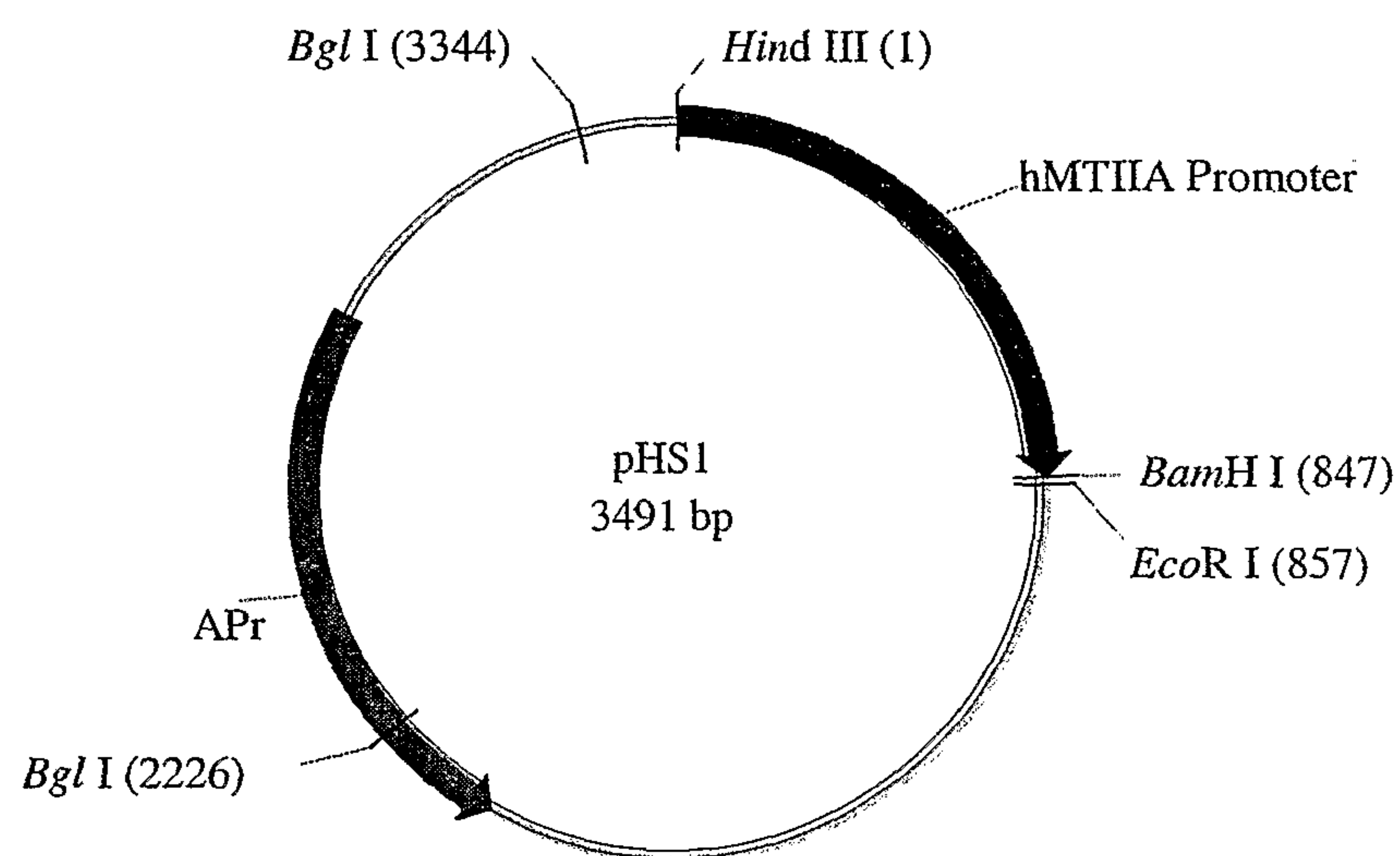
61. A method for the *in vitro* production of an amidated product said method comprising:

- (A) forming a hydroxylated intermediate by reacting a precursor having a glycine residue, in free acid form and attached to a carbonyl group, in the presence of peptidylglycine alpha-hydroxylating monooxygenase; and
- (B) simultaneously or subsequently reacting said intermediate with either a Lewis base or peptidyl alpha-hydroxyglycine alpha-amidating lyase;

wherein said peptidylglycine alpha-hydroxylating monooxygenase has been expressed by the cells or cell lines of claims 34-38.

62. The method of claim 61 wherein step (B) utilizes a Lewis base.

63. A cell line comprising host cells transfected with the vector of claim 7.

Figure 1 Plasmid Map of PHS1**FIG. 1**

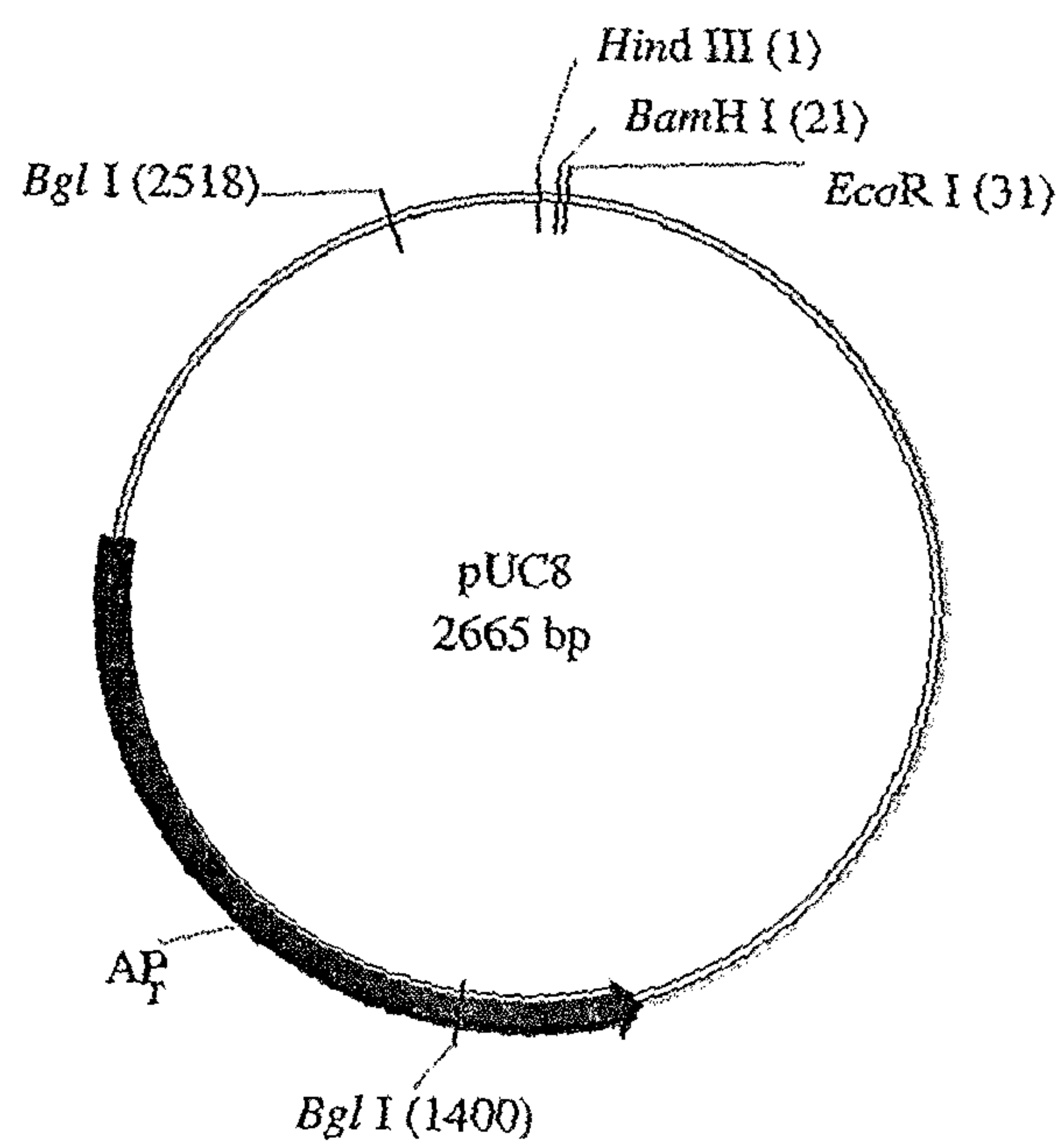
Plasmid Map of pUC8

FIG. 2

Figure 3 Plasmid Map of pSV402MT

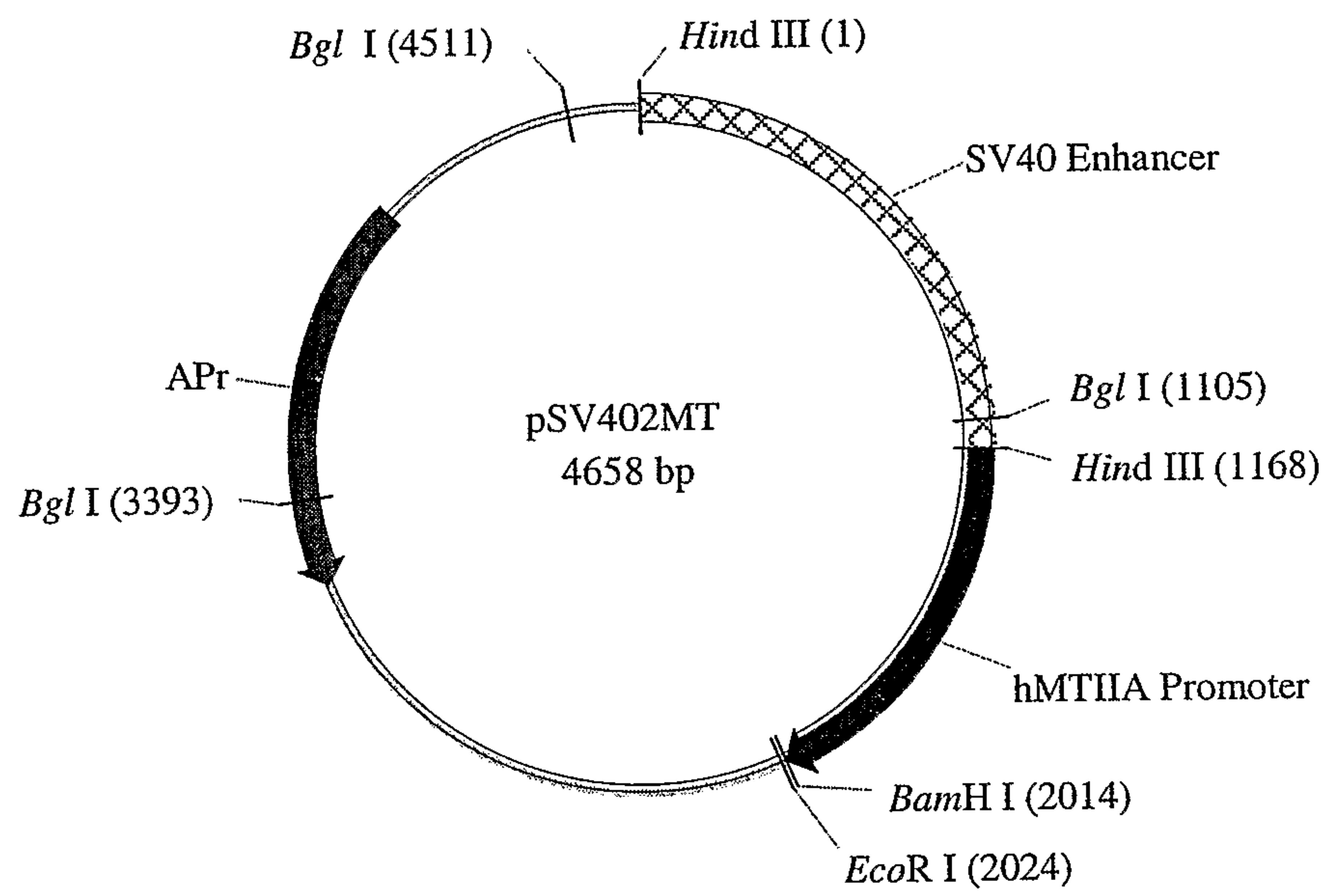


FIG. 3

Figure 4 Derivition of α -AE cDNA Gene Sequence

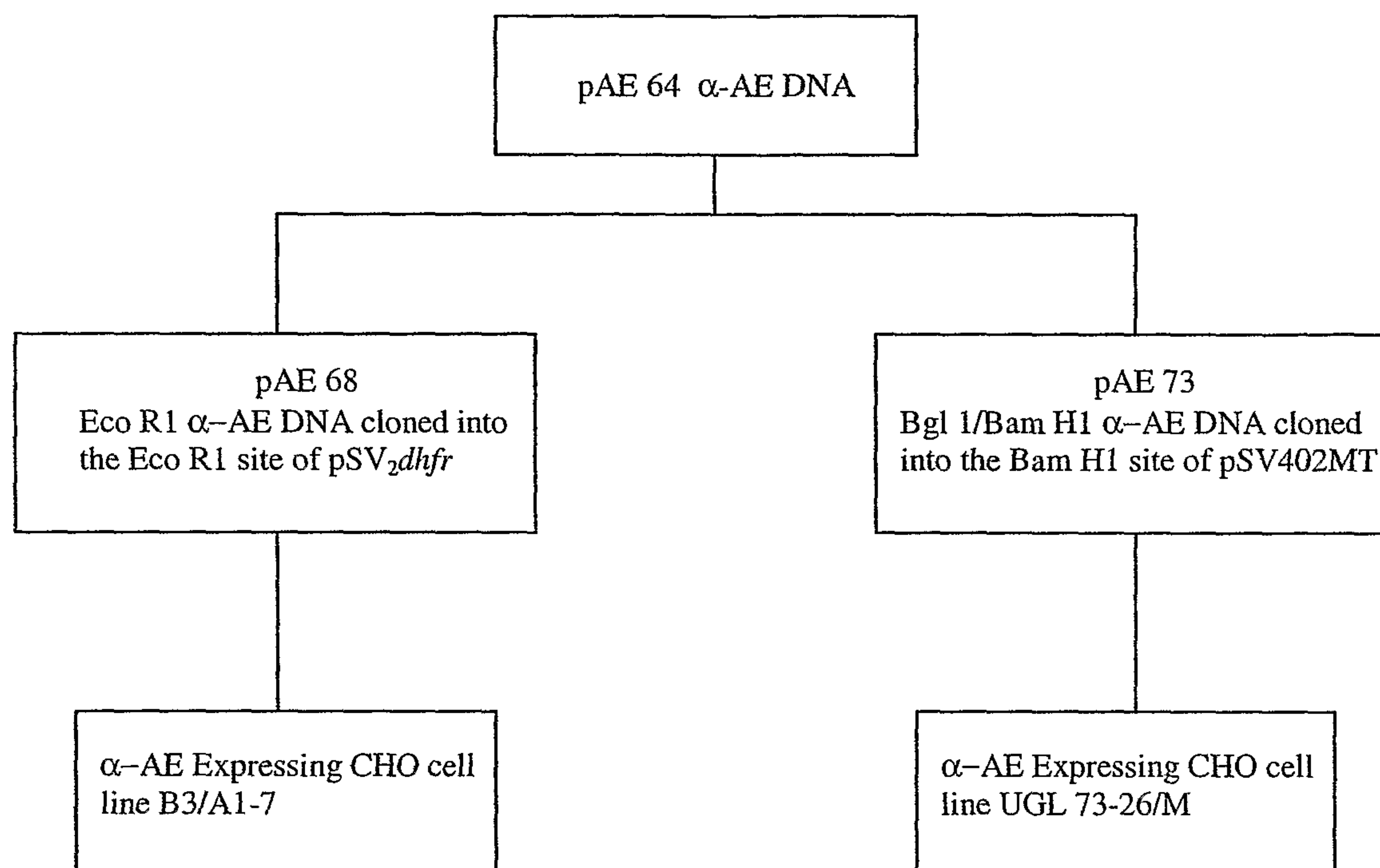


FIG. 4

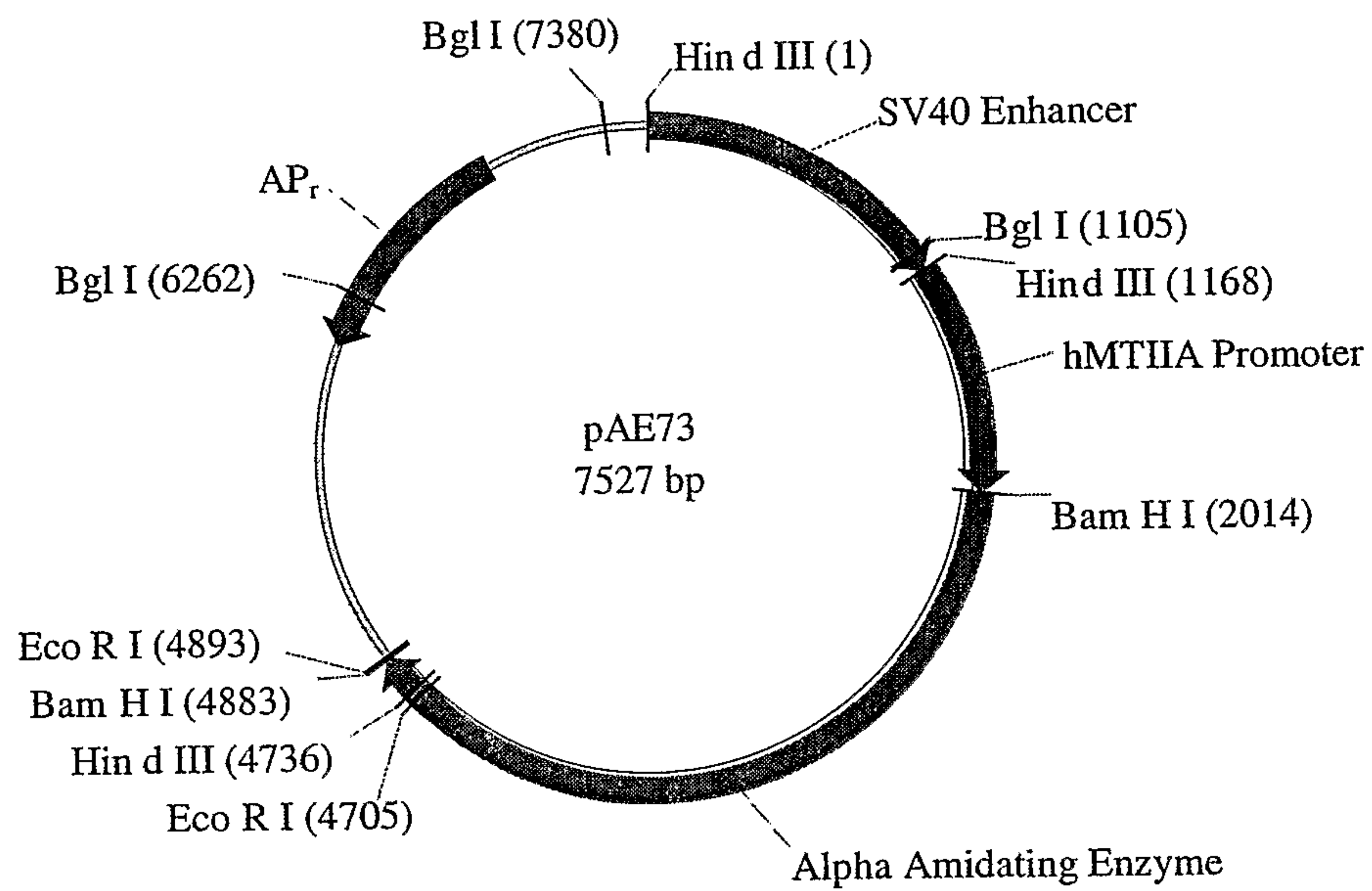
Figure 5 Plasmid Map of pAE73**FIG. 5**

Figure 6 73-26 Suspension Adaptation Scheme

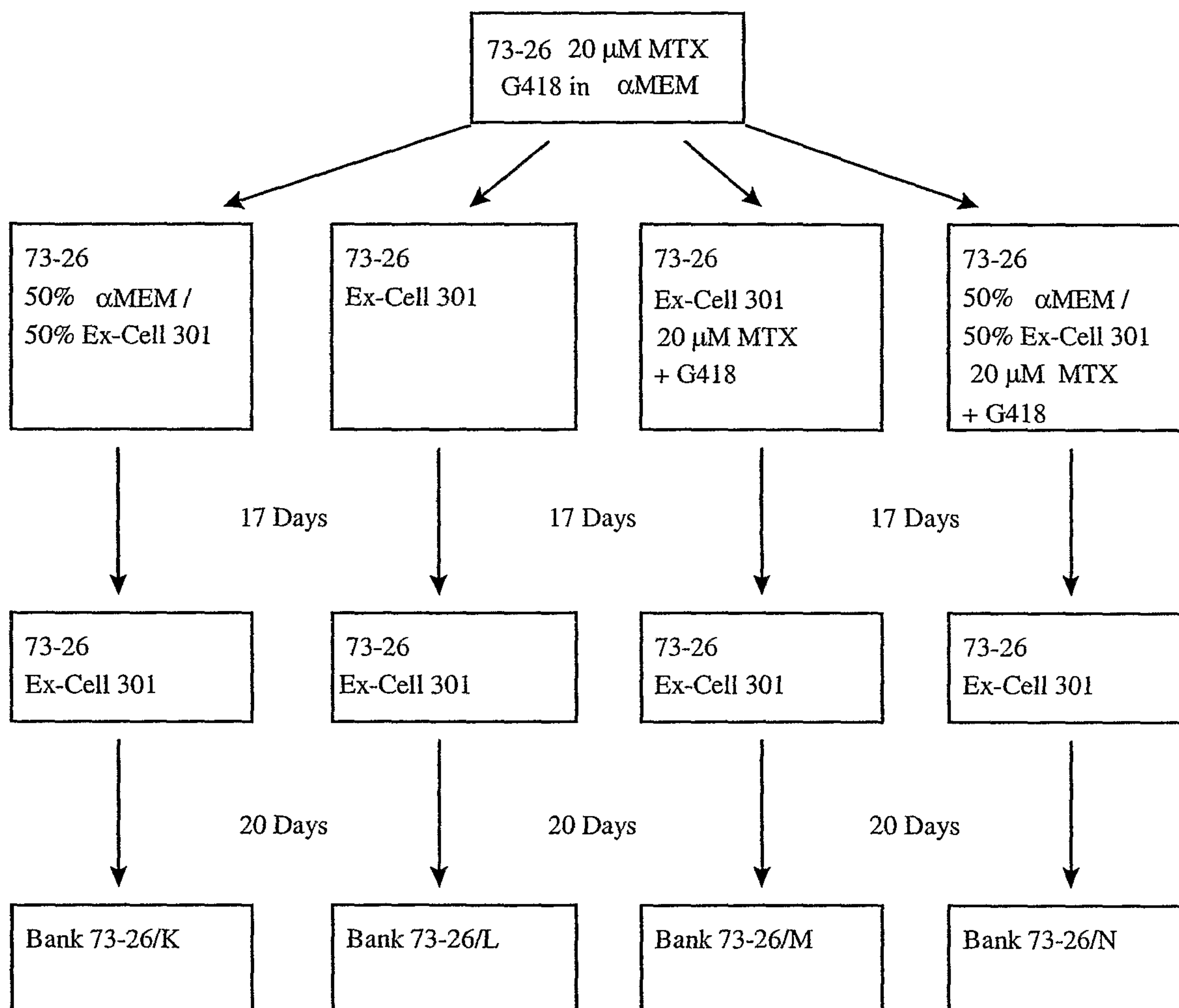


FIG. 6

Figure 7 Dissolved Oxygen Concentration And α -AE Productivity In Spinner Flasks

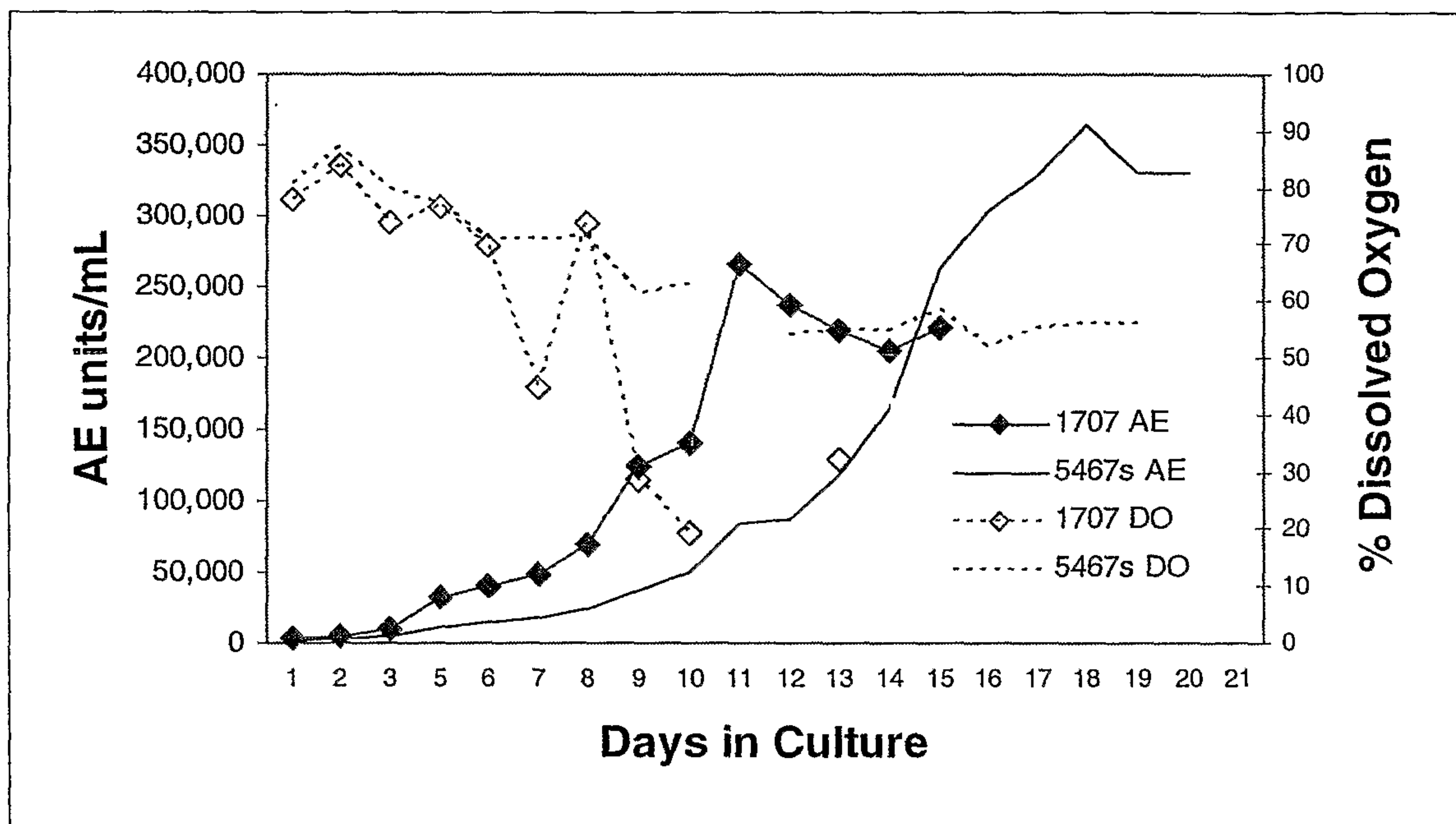


FIG. 7

Figure 8 Effect of Spinner Volume On DO and α -AE Productivity

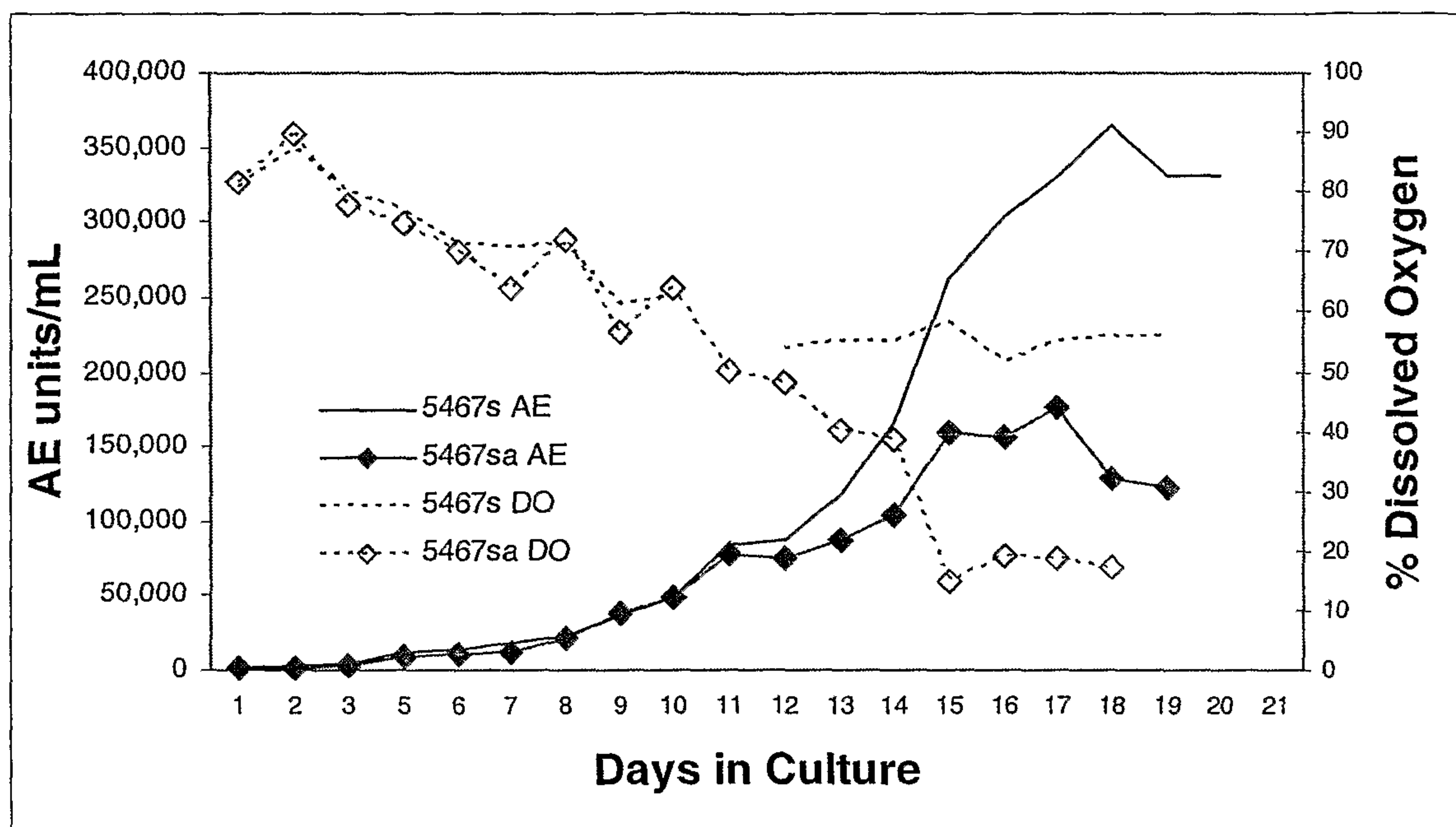


FIG. 8

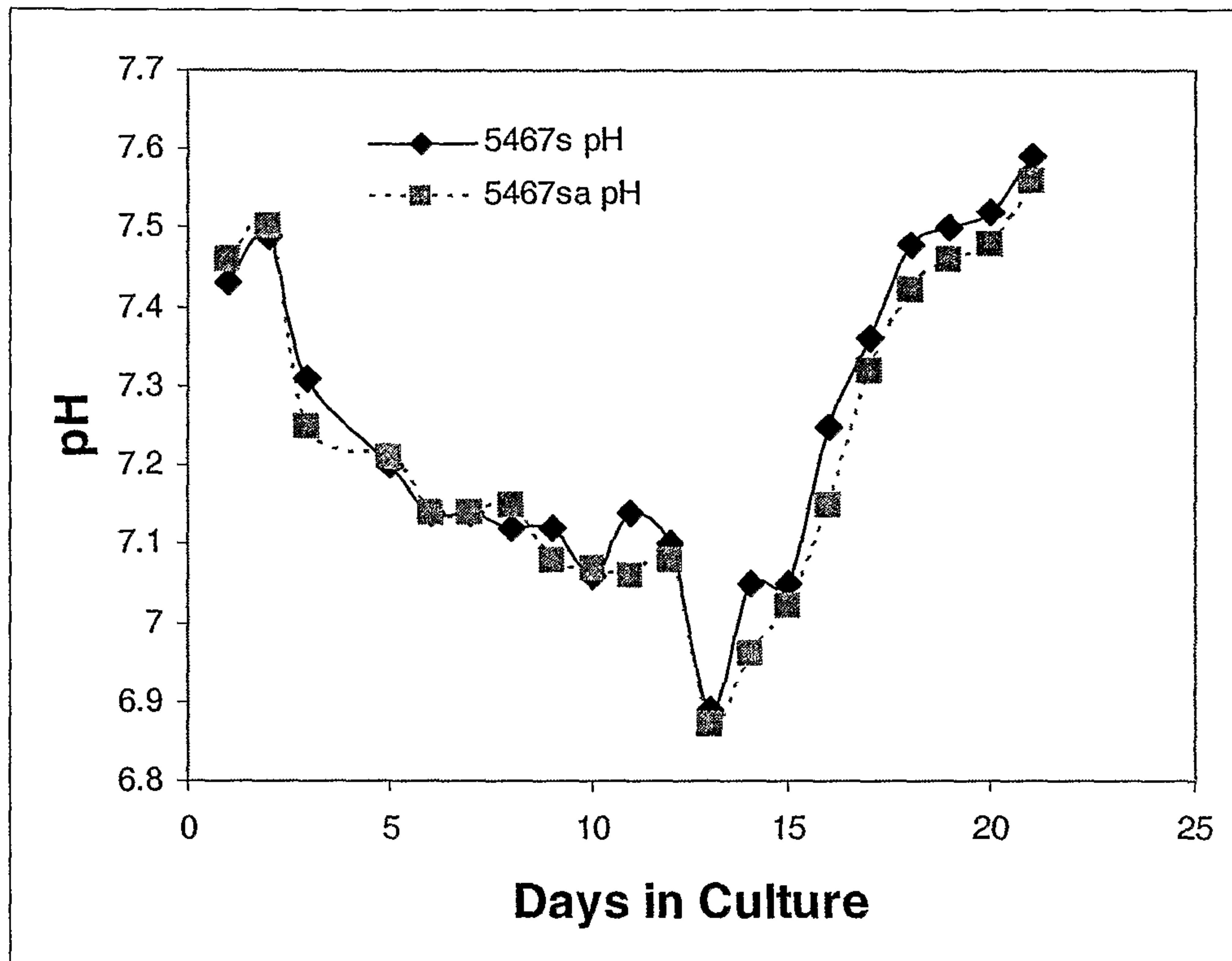
Figure 9 Spinner Culture pH of UGL 73-26/M

FIG. 9

Figure 10 pH Profile Of Stirred Tank Bioreactors And Spinner Flasks Without pH Control

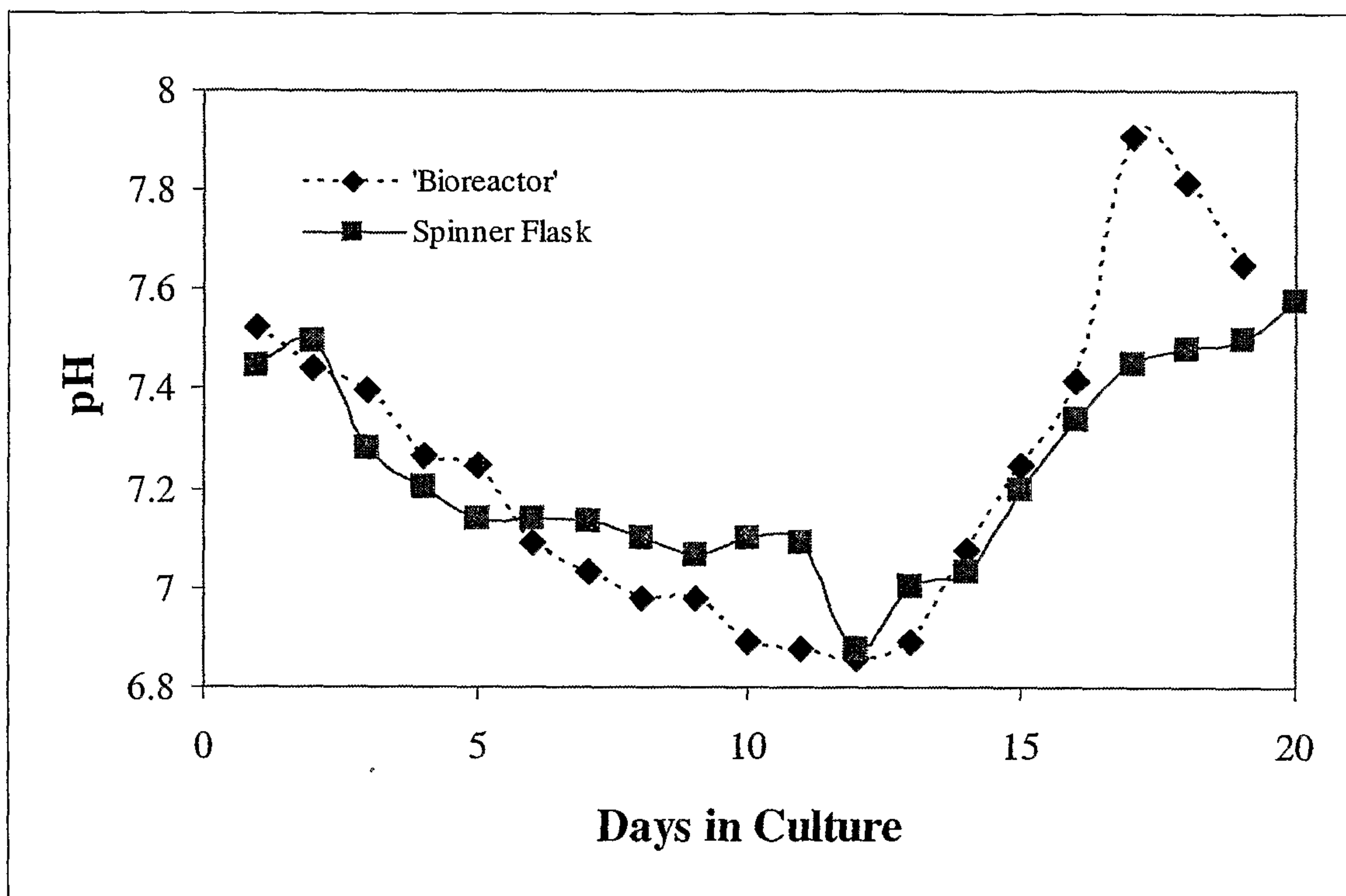


FIG. 10

Figure 11 pH Effect On UGL 73-26/M α -AE Productivity In Stirred Tank Bioreactors

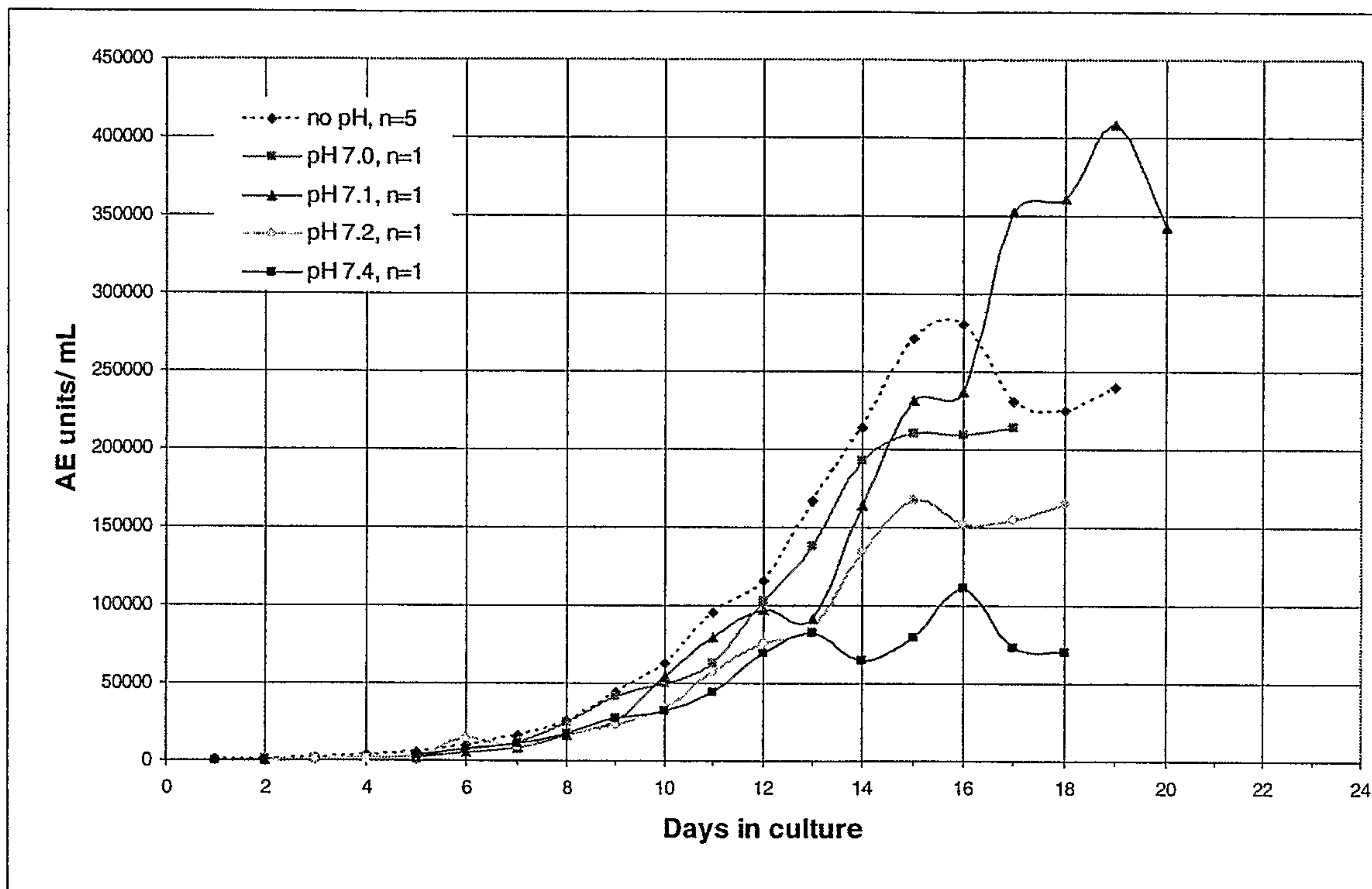


FIG. 11

Figure 12 SDS PAGE Of Purified PAM Enzyme

Lane 1- α -AE reference standard, Lane 2 -Broad Range Molecular Weight Markers, Lane 3 – Clarified Harvest, Lane 4 - TFF 1 retentate, Lane 5 - TFF 1 retentate, Lane 6 – Q-Sepahrose FF eluate, Lane 7 – Q-Sepahrose FF eluate, Lane 8 – Phenyl Sepharose FF eluate, Lane 9 – Phenyl Sepharose eluate, Lane 10 – TFF 2 retentate.

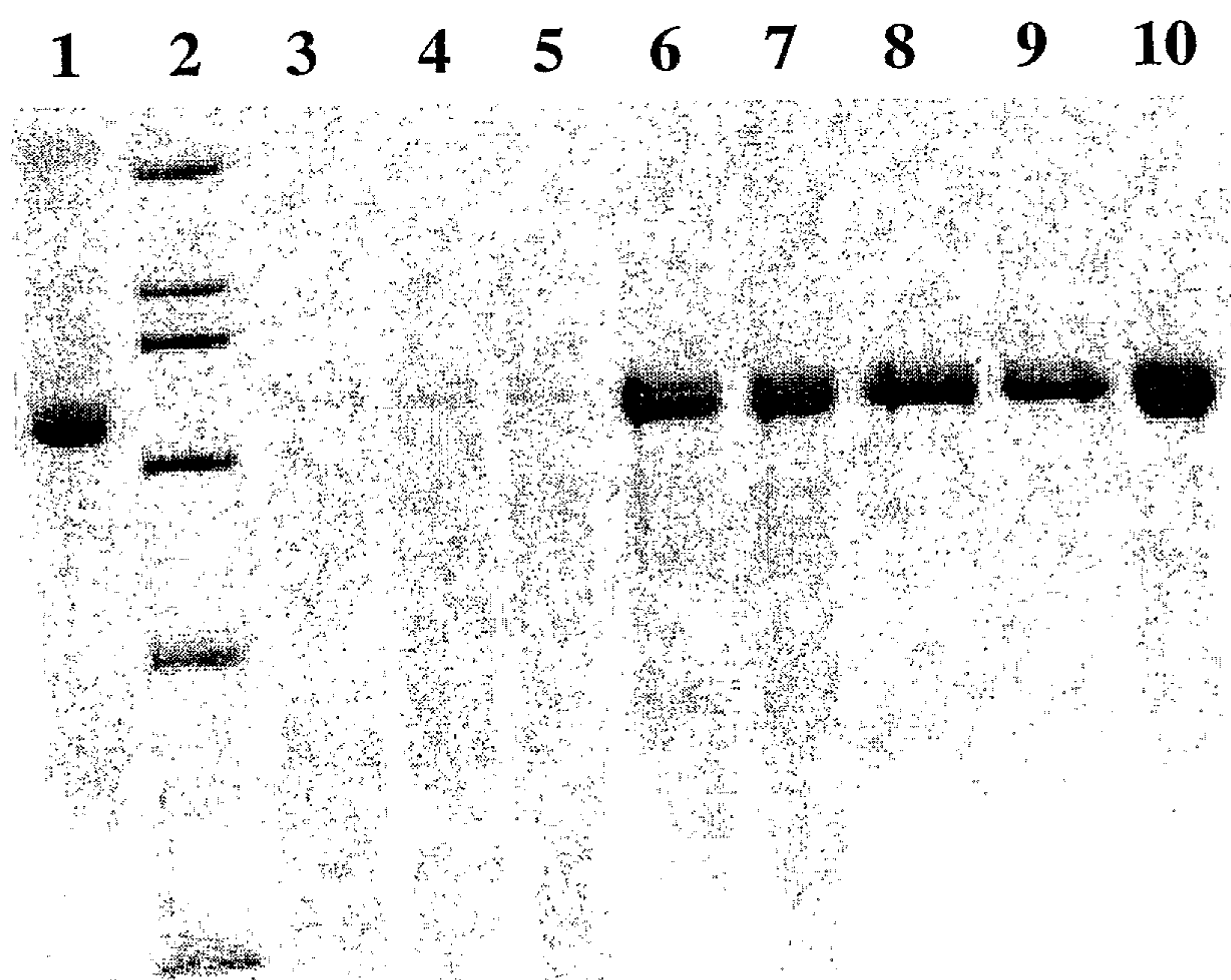


FIG. 12

Figure 13 Densitometry Scan Of SDS PAGE Gel and Calculation of Percent Peak Areas

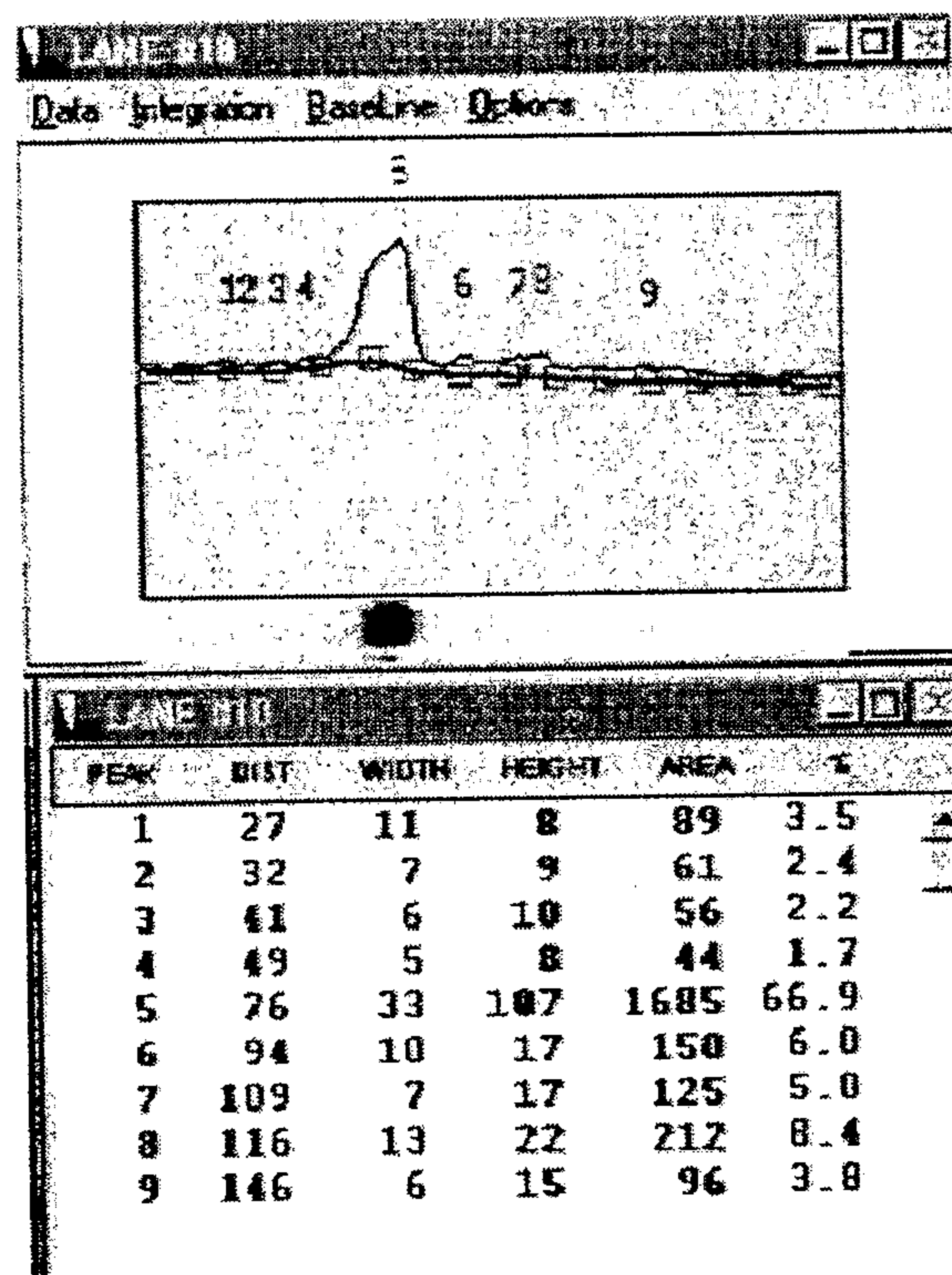


FIG.13

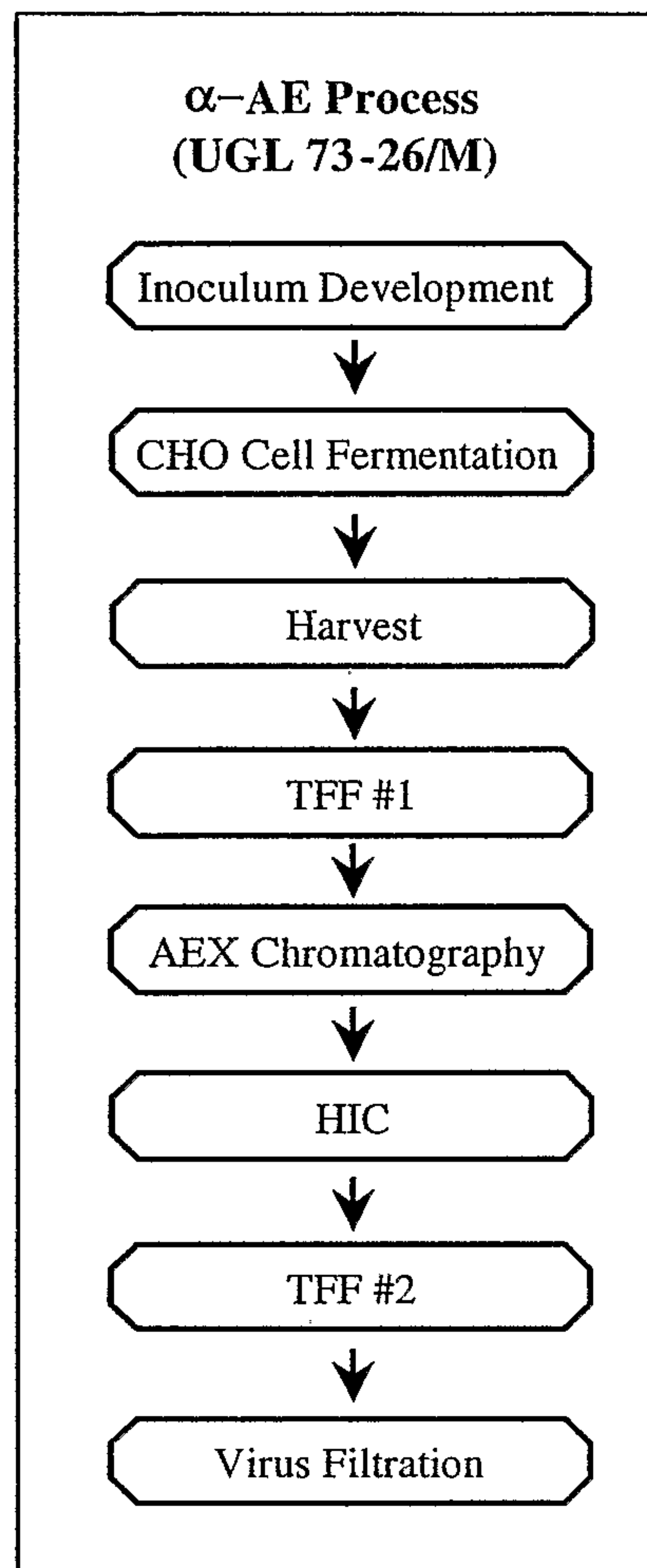
Figure 14 Process Flow Diagram For Production Of α -AE

FIG. 14

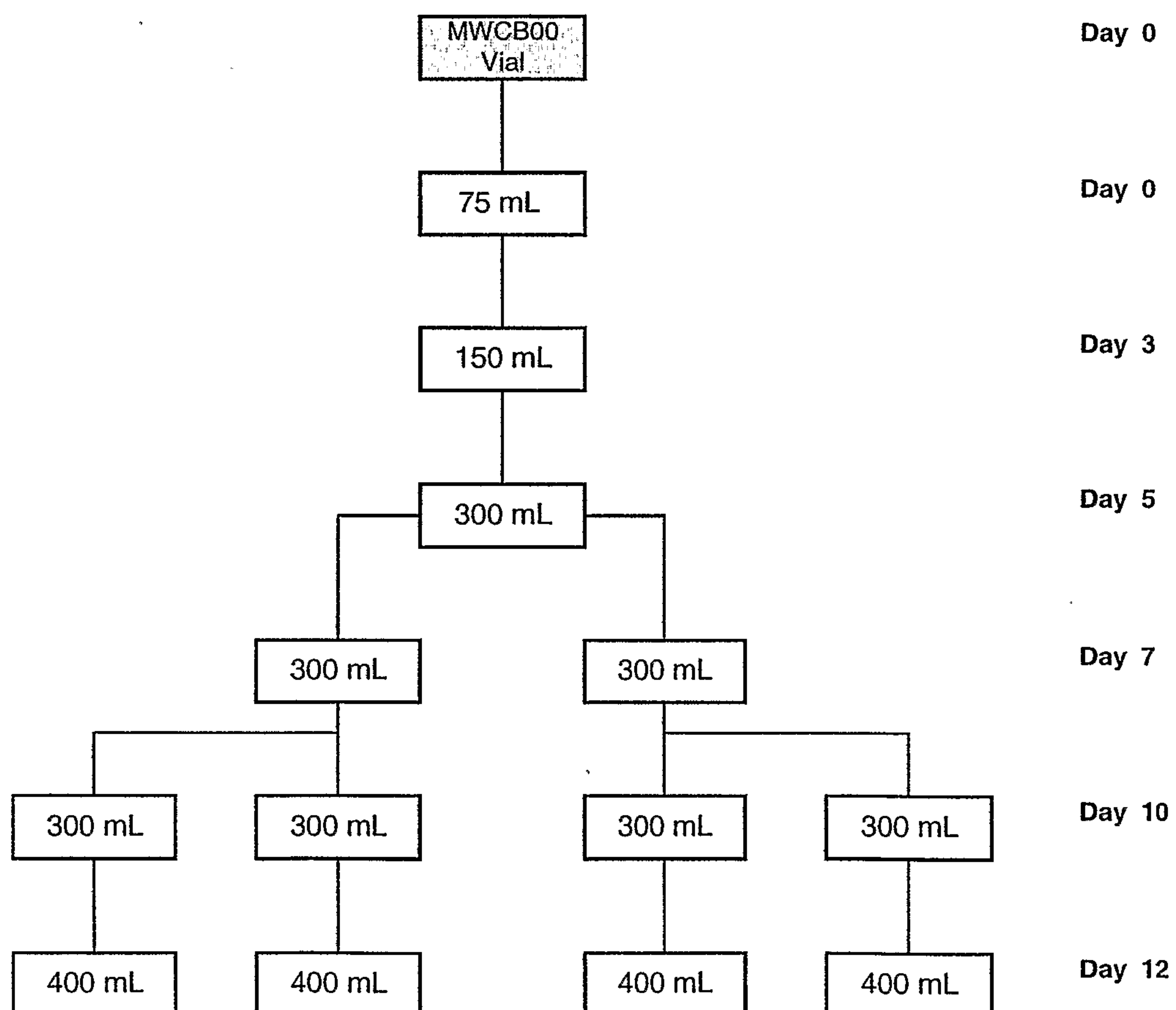
Figure 15 Inoculum Scheme for UGL 73-26/M

FIG. 15

