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CA 2146240 C 2007/05/01

(11)(21) 2 146 240

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 1993/10/06
(87) Date publication PCT/PCT Publication Date: 1994/04/14
(45) Date de délivrance/Issue Date: 2007/05/01
(85) Entrée phase nationale/National Entry: 1995/04/03
(86) N° demande PCT/PCT Application No.: FI 1993/000402
(87) N° publication PCT/PCT Publication No.: 1994/008024
(30) Priorité/Priority: 1992/10/06 (FI924494)

(51) Cl.Int./Int.Cl. *C12N 15/11* (2006.01), *C12N 1/15* (2006.01), *C12N 1/19* (2006.01), *C12N 15/67* (2006.01), *C12N 15/80* (2006.01), *C12N 15/81* (2006.01), *C12N 9/28* (2006.01), *C12P 21/00* (2006.01)

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(54) Titre : PRODUCTION ACCRUE DE PROTEINES SECRETEES PAR DES CELLULES EUCARYOTES RECOMBINANTES

(54) Title: INCREASED PRODUCTION OF SECRETED PROTEINS BY RECOMBINANT EUKARYOTIC CELLS

(57) Abrégé/Abstract:
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CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/80, 15/67, C12P 21/02

A1

(11) International Publication Number: **WO 94/08024**

(43) International Publication Date: 14 April 1994 (14.04.94)

(21) International Application Number: PCT/FI93/00402

(22) International Filing Date: 6 October 1993 (06.10.93)

(30) Priority data:
924494 6 October 1992 (06.10.92) FI

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(81) Designated States: AU, CA, FI, JP, NO, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

2146240

Published
With international search report.

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(57) Abstract

The invention relates to recombinant DNA technology. Specifically this invention relates to new recombinant eukaryotic cells transformed with *SSO* genes. Eukaryotic cells transformed with several copies of *SSO* genes, or overexpressing the Sso protein by some other means, have an increased capacity to produce secreted foreign or endogenous proteins. Further, the said new recombinant cells, when transformed with genes expressing suitable hydrolytic enzymes can utilize appropriate macromolecular compounds more efficiently, which results in increased cell mass production and/or more versatile utilization of the compounds in relevant biotechnical applications.

* (Referred to in PCT Gazette No. 27/1994, Section II)

INCREASED PRODUCTION OF SECRETED PROTEINS BY RECOMBINANT EUKARYOTIC CELLS

Field of the invention

This invention relates to recombinant-DNA-technology. Specifically this invention relates to new recombinant eukaryotic cells transformed with *SSO* genes or their homologues. A eukaryotic cell transformed with several copies of a *SSO* gene or a gene homologous to *SSO* has an increased capacity to produce secreted foreign or endogenous proteins.

Further, the said new recombinant eukaryotic cells, especially yeasts and filamentous fungi, when transformed with genes expressing suitable hydrolytic enzymes can hydrolyze and/or utilize appropriate macromolecular/polymeric compounds more efficiently, which results in increased cell mass production and/or more versatile utilization of the compounds in relevant biotechnical applications.

Background of the invention

The development of recombinant DNA methods has made it possible to produce proteins in heterologous host systems. This possibility greatly facilitates production of e.g. proteins of therapeutic importance which normally occur in nature in very low amounts or are otherwise difficult to isolate or purify. Such proteins include growth factors, hormones and other biologically active proteins or peptides which traditionally have been isolated from human or animal tissues or body fluids e.g. blood serum or urine. The increasing danger of the presence of human pathogenic viruses such as HBV, HIV, and oncogenic viruses or other pathogens in the human or animal tissues or body fluids has greatly speeded up the search for heterologous production systems for these therapeutics. Other proteins of clinical importance are viral or other microbial or human parasite proteins needed for diagnostics and for vaccines especially of such organisms which are difficult to grow *in vitro* or in tissue

culture, or are dangerous human pathogens. These include viruses like HBV, HIV, yellow fever, rubella, FMDV, rabies, and human parasites such as malaria.

A further group of proteins for which heterologous production systems have been or are being developed are secreted enzymes, especially those hydrolyzing plant material, and which are needed in food and fodder production as well as in other industrial processes including textile industry and pulp and paper industry. The possibility of producing proteins in heterologous systems or production of endogenous proteins in genetically engineered cells increases their yields and greatly facilitates their purification and has already by now had a great impact on studies of structure and function of many important enzymes and other proteins. The production and secretion of foreign hydrolytic enzymes in yeast for example, results in improvements in processes based on industrial yeast strains such as distiller's, brewer's or baker's yeasts.

Various production systems have been and are being developed including bacteria, yeasts, filamentous fungi, animal and plant cell cultures and even multicellular organisms like transgenic animals and plants. All of these different systems have their advantages, even if disadvantages, and all of them are needed.

The yeast *Saccharomyces cerevisiae* is at the moment the best known eukaryote at genetic level. As a eukaryotic microbe it possesses the advantages of a eukaryotic cell like most if not all of the post-translational modifications of eukaryotes, and as a microbe it shares the easy handling and cultivation properties of bacteria. The large scale fermentation systems are well developed for *S. cerevisiae* which has a long history as a work horse of biotechnology including production of food ingredients and beverages such as beer and wine.

The yeast genetic methods are by far the best developed among eukaryotes based on the vast knowledge obtained by classical genetics. This made it easy to adopt and further develop for yeast the gene technology procedures first described for *Escherichia coli*. Along other lines the methods for constructing yeast strains

producing foreign proteins have been developed to a great extent (Romanos *et. al.*, 1992).

Secretion of the proteins into the culture medium involves transfer of the proteins through the various membrane enclosed compartments constituting the secretory pathway. First the proteins are translocated into the lumen of the endoplasmic reticulum ER. From there on the proteins are transported in membrane vesicles to the Golgi complex and from Golgi to plasma membrane. The secretory process involves several steps in which vesicles containing the secreted proteins are pinched off from the donor membrane, targetted to and fused with the acceptor membrane. At each of these steps function of several different proteins are needed.

The yeast secretory pathway and a great number of genes involved in it have been elucidated by isolation of conditional lethal mutants deficient in certain steps of the secretory process (Novick *et al.,* 1980; 1981). Mutation in a protein, needed for a particular transfer step results in accumulation of the secreted proteins in the preceding membrane compartment. Thus proteins can accumulate at ER, Golgi or in vesicles between ER and Golgi, or in vesicles between Golgi and plasma membrane.

More detailed analysis of the genes and proteins involved in the secretory process has become possible upon cloning the genes and characterization of the function of the corresponding proteins. A picture is emerging which indicates that in all steps several interacting proteins are functioning. We have recently cloned two new yeast genes, *SSO1* and *SSO2* as multicopy suppressors of *sec1-1* defect in growth and secretion in elevated temperatures (Aalto *et al.*, 1993).

Many of the genes identified in and isolated from *S. cerevisiae* have been found and cloned from other organisms based either on the sequence homology with yeast genes or complementation of yeast mutations. Mammalian NSF factor is the homologue of yeast *SEC18* gene product and displays a similar function in protein secretion (Wilson *et al.*, 1989). *SEC14* gene of *Yarrowia lipolytica* (Lopez *et al.*, 1992) has been cloned and characterized. Mammalian homologue for yeast *SEC11* gene coding for a

component of the signal peptidase has been cloned (Greenberg *et al.*, 1989). *Schizosaccharomyces pombe YPT1* gene coding for a small GTP binding protein was cloned using the yeast gene *SEC4* as a probe (Fawell *et al.*, 1989) and the mammalian counterpart of *YPT1* was shown to be part of the secretory machinery using antibodies against the yeast Ypt1 protein (Segev *et al.*, 1988). Mammalian rab1 protein shown to be homologous to Ypt1p (Zaraoui *et al.*, 1989) can substitute for yeast Ypt1 function (Haubruck *et al.*, 1990).

Genes homologous on the protein level to the yeast *SSO1* and *SSO2* genes according to the invention are found in several species including mouse (Hirai *et al.*, 1992), rat (Inoue *et al.*, 1992, Bennett *et al.*, 1992) and nematode (Ainscough *et al.*, 1991; EMBL Data Bank 29, accession number M 75825) indicating that the genes are conserved during evolution. The homologous proteins in the other species also appear on the cell surface or are implicated to be involved in synaptic vesicle transport to the cell surface, suggesting that they may be functionally related to *SSO1* and *SSO2*. However, direct involvement in secretion has only been demonstrated for the Sso–proteins, reported by us (Aalto *et al.*, 1993). Yeast homologues for the synaptic vesicle membrane proteins, synaptobrevins are the Snc1 and Snc2 proteins (Gerst *et al.* 1992; Protopopov *et al.* 1993).

The above examples, many more of which exist, illustrate the universal nature of the secretory machinery. Results obtained with yeast are largely applicable to other fungi as well as other eukaryotic cells.

Genes with sequence similarity to the *SSO* genes are implicated to function also in other steps of intracellular protein transport/secretion: *SED5* (Hardwick and Pelham, 1992) between ER and Golgi and *PEP12* (Becherer and Jones, 1992) between Golgi and vacuole, the lysosome compartment of yeast. This further supports the central and conserved role of the *SSO* genes in protein secretion and intracellular transport. However, no reports exist so far on any positive effect of the *SSO*–homologues in yeast or animal cells on secretion when overexpressed, which effect we are showing in this invention for the *SSO* genes.

Less is known about the secretory system of other yeasts such as *Kluyveromyces, Pichia, Schizosaccharomyces* and *Hansenula*, which, however, have proven useful hosts for production of foreign proteins (Buckholz and Gleeson, 1991). The genetics and molecular biology of these yeasts are not as developed as for *Saccharomyces* but the advantages of these yeasts as production hosts are the same as for *Saccharomyces*. This holds true also for filamentous fungi such as *Neurospora, Aspergillus* and *Trichoderma* which have been used for production of secreted foreign proteins (Jeenes *et al.*, 1991). Belonging taxonomically to Fungi and very many of the filamentous fungi even belonging to *Ascomycetes*, like *S. cerevisiae* does, it is evident that the secretory machinery of filamentous fungi is similar to that of *S. cerevisiae*. Filamentous fungi are very efficient in secreting their own hydrolytic enzymes. However, production of foreign proteins in filamentous fungi is much less efficient and in many cases this seems to be due to inefficient secretion. The common features of all fungi are for instance post-translational modifications occurring along the secretory pathway.

Several attempts have been made and published previously to increase foreign protein production in yeast and filamentous fungi as well as in other organisms. Much work has been devoted to various promoter and plasmid constructions to increase the transcription level or plasmid copy number (see e.g. Baldari *et al.* 1987; Martegani *et al* 1992; Irani and Kilgore, 1988). A common approach to try and increase secretion is to use yeast signal sequences (Baldari, *et al.* 1987, Vanoni *et al.* 1989). Random mutagenesis and screening for a secreted protein (Smith *et al.,* 1985; Sakai *et al.,* 1988; Schuster *et al.,* 1989; Suzuki *et al.,* 1989; Sleep *et al.,* 1991; Lamsa and Bloebaum, 1990; Dunn-Coleman *et al.*, 1991) or fusion of the foreign protein to an efficiently secreted endogenous protein (Ward *et al.,* 1990; Harkki *et al.*, 1989; Nyyssönen *et al.* 1993; Nyyssönen *et al.*, Pat. Appl.) have been widely used both for yeast and filamentous fungi in order to make the secretion of foreign proteins more efficient. Both of these methods are of limited use. Overproduction mutants isolated by random mutagenesis and screening are almost exclusively recessive and thus cannot be transferred into industrial yeast strains which are polyploid. Often the overproduction results from changes other than increased secretion and in many cases

affects only the protein used for screening. Fusion protein approach requires tailoring of the fusion construction for each foreign protein separately.

Our approach, increasing the copy number of genes functioning in secretion and thus the amount of components of the secretory machinery is more universal: it is applicable to any protein without specific fusion constructions and applicable to diploid and polyploid strains.

It is not exactly known which steps form the bottle necks in the secretory process, but it can be anticipated that there are several ones of them. We started to unravel the potential blocks at the very end of the secretory pathway, and have cloned and characterized genes participating at the very final stage of the secretory process at which the secretory vesicles budding from the Golgi complex are targetted to and fused with the plasma membrane to release the secreted proteins to the cell exterior. We have previously cloned and characterized *SEC1* functioning at this stage (Aalto *et al.,* 1991; Aalto *et al.,* 1992) and have later shown that *SEC1* is an essential single copy gene (Aalto *et al.,* 1993). The *SSO* genes according to the invention were cloned as multicopy suppressors of *sec1-1* mutation (Aalto *et al.*, 1993).

Summary of the invention

The present invention describes the isolation of genes which, when overexpressed enhance the production of secreted proteins. Specifically, the present invention describes the isolation of *SSO1* and *SSO2* genes of *S. cerevisiae* coding for Sso1p and Sso2p, respectively, the characterization of the genes and their transfer into, and overexpression in *S. cerevisiae.* In addition, this invention describes isolation of a *SSO* homologue from *Trichoderma reesei*, characterization of the gene, and transfer and overexpression in *Trichoderma*.

Furthermore, the sequence homologies between the yeast *SSO* genes and their higher eukaryotic counterparts indicates that this invention can be used to construct novel cell lines for higher eukaryotes with increased secretion capacity.

This invention thus provides new recombinant eukaryotic cells, preferably fungal host cells expressing enhanced levels of Sso protein(s), and especially yeast strains expressing enhanced levels of Sso1 and/or Sso2 proteins as well as *Trichoderma* strains expressing enhanced levels of *Trichoderma* Sso–protein. This invention also provides process(es) for production of increased amounts of secreted proteins by overexpressing genes interacting with the *SSO* genes, such as *SEC1*.

The eukaryotic cells according to the invention being transformed with the *SSO* genes or genes interacting with the *SSO* genes have an increased capacity to produce secreted proteins. The new eukaryotic cells according to the invention, especially yeast and filamentous fungi, can also be used for more efficient production of hydrolytic enzymes and hydrolysis of e.g. polymeric substrates which results in improvements in biotechnical processes such as single cell or baker's yeast production due to increased cell mass or in other processes where efficient production of hydrolytic enzymes and/or efficient hydrolysis of plant material is beneficial.

Brief description of the drawings

Figs. 1A and 1B show the *S. cerevisiae SSO1* and *SSO2* gene cDNA integrated into a multicopy plasmid pMAC561 resulting in plasmids YEpSSO1 and YEpSSO2, respectively.

Fig. 2 shows Western analysis demonstrating overexpression of Sso2 protein in yeast transformed with YEpSSO2.

Fig. 3 shows increased production of secreted *Bacillus* α–amylase by *S. cerevisiae* (strain sf750–14D) transformed with multicopy plasmid, expressing *SSO1* or *SSO2* gene and with another plasmid expressing *Bacillus* α–amylase gene.

Fig. 4 shows Western analysis of *Bacillus* α–amylase secreted by *S. cerevisiae* with or without the multicopy plasmid expressing *SSO1* or *SSO2* gene.

Fig. 5 shows increased production of secreted *Bacillus* α–amylase by *S. cerevisiae* (strain DBY746) transformed with multicopy plasmid, expressing *SSO2* gene and with another plasmid expressing *Bacillus* α–amylase.

Fig. 6 shows increased production of secreted *Bacillus* α–amylase by *S. cerevisiae* (strain DBY746) transformed with a multicopy plasmid expressing *SEC1* gene and with another plasmid expressing *Bacillus* α–amylase.

Fig. 7 shows the *SSO2* expression cassette flanked by ribosomal sequences integrated into BS+, generating the vector pRbSSO2.

Fig. 8 shows hybridization of DNA derived from six different fungal species with the yeast *SSO1* gene.

Detailed description of the invention

For better understanding of the following detailed description of the invention it may be helpful to give definitions of certain terms to be used hereinafter.

Overexpression of a gene: The protein encoded by the said gene is produced in increased amounts in the cell. This can be achieved by increasing the copy number of the gene by introducing extra copies of the gene into the cell on a plasmid or integrated into the genome. Overexpression can also be achieved by placing the gene under a promoter stronger than its own promoter. The amount of the protein in the cell can be varied by varying the copy number of the gene and/or the strength of the promoter used for the expression.

Suppression of a mutation: When the effect of a mutation in a given gene is alleviated or abolished by a mutation in an other gene, this second gene is called a suppressor of the first gene. Suppression can occur also by overexpression of the wild type allele of the second gene by the means described above. This is called overexpression suppression. If the overexpression is caused by multiple copies of the

suppressing gene the suppression can also be called multicopy suppression. Suppression phenomenon indicates that these two genes interact at genetic level. The interaction may also occur at physical level as direct, physical contact between the two proteins encoded by the interacting genes.

Homologous genes, homologues: Genes which are related, but not identical, in their DNA sequence and/or perform the same function are homologous with each other and are called each other's homologues.

Secreted proteins: Proteins which inside of the cell are directed to the secretory pathway and transported through it to the exterior of the cell, outside of the plasma membrane, are called secreted proteins. In yeast the proteins may remain associated with the cell wall such as invertase or released through the cell wall into the growth medium such as the foreign protein *Bacillus* α–amylase.

SSO1 and *SSO2* genes to be used in this invention are isolated from an organism containing these genes e.g. *Saccharomyces cerevisiae* and *Trichoderma* spp. Also other suitable yeasts and other fungi, such as *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Pichia* spp., *Hansenula* spp., *Aspergillus* spp., *Neurospora* spp. and *Penicillium* spp. can be used. It is to be noted that homologous genes from other organisms can also be used.

Furthermore, overexpression of other genes functioning at the same step with the *SSO* genes, such as *SEC1*, in the presence of normal or increased levels of Sso–proteins results in increased secretion. Genes functioning at the preceding steps of the secretory process may well have a similar effect. Thus, release of the secretory vesicles from the Golgi compartment may be facilitated by increasing the copy number of *SEC7* and/or *SEC14* genes known to function at this step (Novick *et al.* 1980) or by searching for and increasing the copy number of genes interacting with *SEC7* and/or *SEC14* e.g. suppressors of their mutations. Likewise any previous step of the secretory process may be improved by increasing the copy number of genes involved. The new genes we have isolated from *S. cerevisiae*, *SSO1* and *SSO2*

represent duplicated genes which suggests that they play an important role in the cell. Based on the conserved nature of *SSO1* and *SSO2* and their homologues in other species, as mentioned above, we propose that increase of the *SSO* genes in any other eukaryotic species would result in increased protein secretion efficiency including other yeasts, filamentous fungi, and plant and animal cells.

It is to be noticed that due to the fact that many genes involved in secretion function in other organisms, this invention covers for instance also expression of yeast genes in filamentous fungi and higher eukaryotes and *vice versa*, or any eukaryotic gene in another eukaryote to obtain enhanced secretion.

The host to be transformed with the genes of the invention can be any eukaryotic cell suitable for foreign or endogenous protein production, e.g. any *S. cerevisiae* yeast strain, (e.g. DBY746, AH22, S150-2B, GPY55-15Bα, VTT-A-63015) any *Trichoderma* spp. such as *T. harzianum* and the *T. reesei* strains derived from the natural isolate QM6a, such as RUTC-30, QM9416 and VTT-D-79125, any *Kluyveromyces s*pp., *Sch. pombe, H. polymorpha, Pichia, Aspergillus, Neurospora, Yarrowia, Penicillium* spp. or higher eukaryotic cells. Transfer of the genes into these cells can be achieved, for instance, by using the conventional methods described for these organisms.

The DNA sequence containing *SSO1* or *SSO2* is isolated from *S. cerevisiae* by conventional methods. In a preferred embodiment gene or cDNA library on a multicopy plasmid is used to suppress the temperature-sensitivity of *sec1-1* mutant (Aalto *et al.*, 1991; 1993) or mutations leading to deficiency in the *SSO* function of *S. cerevisiae* or analogous mutations of other species. In another approach the known DNA sequence of the *SSO* genes and *SSO*-like genes is used to design probes for heterologous hybridization or PCR primers for cloning the *SSO* genes. In still another approach antibodies to the known *SSO* and *SSO*-like genes are used for cloning the gene by standard methods.

The genes corresponding to the *S. cerevisiae SSO1* and *SSO2* are isolated from the other fungi or higher eukaryotes with one or several of the following methods, which are here described specifically for the filamentous fungus *Trichoderma reesei* and which can be modified according to conventional knowledge and means to suit the eukaryotic cell in question.

A cDNA bank of *T. reesei* is constructed into the yeast vector pFL60 as described in the FI patent application No. 92 2373 (Buchert *et al.*). This gene bank DNA is transformed into the *S.cerevisiae* strain H458 (Aalto *et al.*, 1993) and screened for complementation of the secretion defect e.g. as described in Example 6. The plasmid is isolated from the positive colonies and the gene is isolated and characterized using standard methodology, and the corresponding chromosomal gene is isolated. Succesful complementation shows that functionally equivalent genes to the yeast *SSO* genes exist in other fungi such as *T. reesei*.

Alternatively, the genes encoding proteins corresponding to the *S. cerevisiae* Sso1p and/or Sso2p can be isolated from a cDNA or a chromosomal gene bank prepared from *T. reesei* by heterologous hybridization in non-stringent conditions as described in Example 7 and characterized by conventional methods and their function can be shown as described above. Similar approach is suitable for all organisms which have shown to possess chromosomal sequences homologous to the yeast *SSO* genes as analyzed for instance by Southern hybridization of total DNA. It is also possible that the gene can be isolated from an expression library with antibodies prepared against the yeast Sso proteins.

Alternatively, oligonucleotide primers can be designed based on the homologies found between the sequences of the corresponding genes isolated from several organisms. Clear homologies are seen for instance in regions extending from aa 266 to aa 287 in Sso1p and from aa 269 to aa 290 in Sso2p, shown in SEQ ID NO. 1 and SEQ ID NO. 3, respectively. These primers are used to amplify the *T. reesei* gene in a PCR reaction.

To construct a plasmid suitable for transformation into a yeast, the *SSO1* or *SSO2* gene is cloned into a suitable yeast expression vector, such as pAAH5 (Ammerer, 1983) or vectors derived from it (Ruohonen *et al.*, 1991; Ruohonen *et al.*, manuscript in preparation, a) comprising the appropriate yeast regulatory regions. These regulatory regions can be obtained from yeast genes such as the *ADH1, GAL1 – GAL10, PGK1, CUP1, GAP, CYC1, PHO5*, or asparagine synthetase gene, for instance. Alternatively, also the regulatory regions of *SSO1* or *SSO2* can be used to express the genes in *S. cerevisiae*. The plasmid carrying the *SSO1* or *SSO2* gene is capable of replicating autonomously when transformed into the recipient yeast strain. The gene *SSO1* or *SSO2* together with the appropriate yeast regulatory regions can also be cloned into a single copy yeast vector such as pHR70 of Hans Ronne or pRS313, pRS314, pRS315 or pRS316 (Sikorski and Hieter, 1989).

Alternatively, extra copies of *SSO1* or *SSO2* gene can also be integrated into the yeast chromosome, into the ribosomal RNA locus, for instance. For this purpose the ribosomal sequences of a suitable plasmid, e.g. plasmid pIRL9 (Hallborn *et al.*, Pat. Appl.) are released, and cloned appropriately into BS+ vector, as shown in Fig. 7. The gene *SSO1* or *SSO2* coupled in between suitable yeast promoter and terminator regions, is released from the hybrid vector comprising the gene and cloned into the plasmid obtained at the previous stage. From this resulting plasmid the expression cassette, flanked by ribosomal sequences can be released. This fragment is cotransformed into a yeast with an autonomously replicating plasmid carrying a suitable marker for transformation. The plasmid can be later on removed from the cells containing the extra copies of *SSO1* or *SSO2* gene integrated in the chromosome by cultivating the cells in non–selective conditions. This way, recombinant strains can be obtained which carry no extra foreign DNA such as bacterial vector sequences. If a polyploid yeast strain, such as VTT–A–63015, is used the gene can be integrated also to an essential locus such as the *ADH1* or the *PGK1* locus.

To express the *SSO* genes in *Trichoderma* the coding region of the *Trichoderma sso* gene is coupled for instance between the *T. reesei cbh1* promoter and terminator and the expression cassette is transformed into a *Trichoderma* strain producing for

instance mammalian antibodies or another foreign protein or into a strain producing EGIcore, another cellulase or a hydrolytic enzyme. Enhancement of secretion would be especially desired when the fungus is grown on glucose–containing media and for this purpose the *sso* gene(s) need to be expressed from constitutive promoters or promoters functioning on glucose medium.

For filamentous fungi the *sso* gene is preferably integrated into the genome using methods known in the art. Suitable promoters in addition to the *cbh1* promoter or promoter of the *sso* gene itself are for instance the other cellulase promoters, *cbh2, egl1, egl2*, or *tef1, pgk, gpd, pki*, the glucoamylase, α–amylase or the alcohol dehydrogenase promoter. In filamentous fungi transformation usually results in strains with varying copies of the *sso* gene integrated into the genome (Penttilä *et al.*, 1987) and from these the strain with optimal level of *sso* expression for growth and enhanced secretion can be screened.

An object of this invention is thus to provide *SSO* genes, especially the *SSO1* and *SSO2* genes of *S. cerevisiae*, as well as homologous gene(s) of *Trichoderma reesei* and other eukaryotic cells. The sequence of the genes can be determined from the plasmids carrying them by using e.g. the double stranded dideoxy nucleotide sequencing method (Zagursky *et al.*, 1986). The sequence of the *SSO1* gene of *S. cerevisiae* is given as the SEQ ID NO. 1 and the sequence of the *SSO2* gene of *S. cerevisiae* is given as the SEQ ID NO. 3.

Another object of this invention is to provide specific vectors comprising the *SSO* genes. For yeast such a vector is either an autonomously replicating multicopy or a single copy plasmid or a vector capable of integrating into the chromosome, as described above. For *Trichoderma* such a vector is preferably a plasmid from which the expression cassette (promoter – gene – terminator) can be released by restriction enzymes to be integrated into the fungal genome.

Still another object of this invention is to provide yeast or other fungal strains as well as eukaryotic cell lines containing extra copies of *SSO* genes either on replicating

plasmid(s) or integrated into the chromosomes, which results in increased production of secreted proteins, such as yeast invertase or *Trichoderma* cellulases or other hydrolases.

Thus a method for constructing new eukaryotic cells capable of expressing enhanced levels of Sso protein(s) comprises:

(a) isolating DNA sequence(s) coding for Sso protein(s) from a suitable donor organism;

(b) constructing vector(s) carrying at least one of the said DNA sequences; and

(c) transforming at least one of the vectors obtained to suitable host cells.

Still another object of this invention is to provide eukaryotic cells which in addition to extra copies of *SSO* genes comprise a DNA sequence coding for a secreted foreign or endogenous protein, such as α–amylase, cellulase, or an antibody and are capable of expressing this protein.

Thus a process for producing increased amounts of secreted foreign or endogenous protein(s) by overexpressing the *SSO* gene(s) is provided. This process comprises:

(a) isolating DNA sequence(s) coding for the said protein(s) from a suitable donor organism;

(b) constructing a vector carrying at least one of the said DNA sequences;

(c) transforming the vector obtained into a suitable host expressing enhanced levels of Sso protein(s) to obtain recombinant host cells; or alternatively, transforming the vector to a suitable host and retransforming this transformant with *SSO* or a gene homologous to *SSO* and screening for cells with enhanced production of the said protein(s); and

(d) cultivating said recombinant host cells under conditions permitting expression of said protein(s).

A further object of this invention is to improve secretion by optimizing the Sso–protein level using different promoters and different copy numbers of the gene and combining the *SSO* genes with other genes involved in secretion, such as *SEC1*.

Thus the invention provides a process for producing increased amounts of secreted foreign or endogenous protein(s), by overexpressing gene(s) interacting with the *SSO* gene, e.g. *SEC1*, in the presence of normal or increased amounts of the Sso protein(s), which process comprises:

(a) isolating DNA sequence(s) coding for the said protein(s) from suitable donor organism;

(b) constructing a vector carrying at least one of the said DNA sequences;

(c) transforming the vector obtained into a suitable host expressing normal or enhanced levels of Sso protein(s) and overexpressing other gene(s) interacting with *SSO* gene, e.g. *SEC1*, to obtain recombinant host cells; or, alternatively, transforming the vector to a suitable host and retransforming this transformant with *SSO* or a gene homologous to *SSO* and by the gene interacting with *SSO* gene and screening for cells with enhanced production of the said protein(s); and

(d) cultivating said recombinant host cells under conditions permitting expression of said protein(s).

Still another object of this invention is to provide a process for increased production of an endogenous secreted protein, the process comprising:

(a) transforming cells producing the said protein with a *SSO* gene or a gene homologous to *SSO*, alone or together with gene(s) interacting with the *SSO* gene, such as *SEC1*,

(b) screening for transformants producing enhanced level of the said protein thus obtaining recombinant cells for enhanced protein production, and

(c) cultivating said recombinant cells in conditions permitting expression of said protein.

Still another object of this invention is to provide fungal strains which in addition to extra copies of *SSO* genes or their homologue comprise DNA sequence(s) coding for hydrolytic enzyme(s) such as α–amylase and/or glucoamylase or lignocellulose hydrolyzing enzymes such as cellulase(s), hemicellulases or ligninases, which render

the fungus capable of increased hydrolysis of, and/or enhanced growth on polymeric compounds such as starch or lignocellulose.

Thus an efficient biomass production on said raw material or efficient hydrolysis of said raw material is provided. This process comprises:

(a) isolating DNA sequence(s) coding for endogenous or foreign hydrolytic enzyme(s) from a suitable donor organism;

(b) constructing a fungal vector carrying at least one of the said DNA sequences;

(c) transforming the vector obtained into a suitable fungal host expressing enhanced levels of Sso protein(s) to obtain recombinant host cells; or alternatively, transforming the vector to a suitable host and retransforming this transformant with *SSO* or a gene homologous to *SSO* and screening for cells with enhanced production of the said enzyme(s); and

(d) cultivating said recombinant host cells under conditions permitting expression of said hydrolytic enzyme(s).

A process is also provided for efficient biomass production on a raw material or efficient hydrolysis of a raw material, by overexpressing genes interacting with the *SSO* gene, e.g. *SEC1*, in the presence of normal or increased amounts of the Sso protein(s). This process comprises:

(a) isolating the DNA sequence(s) coding for endogenous or foreign hydrolytic enzyme(s) from a suitable donor organism;

(b) constructing a vector carrying at least one of the said DNA sequences;

(c) transforming the vector obtained to a suitable host expressing enhanced levels of proteins interacting with the Sso protein(s) in the presence of normal or increased amounts of the Sso protein(s) to obtain recombinant host cells, or, alternatively, transforming the vector to a suitable host and retransforming this transformant with *SSO* gene or a gene homologous to *SSO* and with the gene(s)

interacting with *SSO* gene, such as *SEC1*, and screening for cells with enhanced production of the said enzyme(s); and

(d) cultivating said recombinant host cells under conditions permitting expression of said hydrolytic enzyme(s).

Possible applications of said recombinant cells are e.g. in single cell production, improved alcohol production or in processes where efficient hydrolysis of raw material is desired.

EXPERIMENTAL

Example 1: Cloning of the coding region of *SSO1* and *SSO2* genes from *Saccharomyces cerevisiae*.

The *SSO1* and *SSO2* genes were isolated as suppressors of the temperature-sensitive defect of *sec1-1* mutant (Novick and Scheckman, 1979; Novick *et al.*, 1980). The *S. cerevisiae* strain sf750-14Dα (α *sec1-1 his4 ura3-52 trp1-289 leu2-3 leu2-112*) (obtained from Randy Scheckman, University of California, Berkeley, CA) was transformed (Ito *et al.*, 1983) by yeast cDNA library constructed by McKnight and McConaughy (1983) from strain X2180-1B on a 2μ based plasmid, pMAC561, containing *TRP1* as a selection marker, and selected for Trp-prototrophy at 37°C. As the growth of the transformants was refractory at 37°C, further work was done at 36.5 or 35°C temperatures which still are non-permissive for *sec1-1*. DNA isolated (Keränen, 1986) from four yeast transformants which showed co-segregation of the Trp^+ phenotype and growth at 36.5°C was transferred into *E. coli* (Hanahan, 1983). Plasmid DNA isolated from *E. coli* transformants was used to re-transform the *sec1-1* strain of *S. cerevisiae*.

Efficient transformation for growth at 36.5°C was obtained. Restriction enzyme analysis of the plasmids indicated that two different sequences were recovered from the cDNA library used. The insert DNA from the two different clones, 1 and 7, was sequenced using the double stranded dideoxy method (Zagursky *et al.*, 1986) and

suitable subclones constructed with standard recombinant DNA methods (Maniatis *et al.,* 1982) or specific primers. The two clones contained an open reading frame of 870 nucleotides (clone 1) and 885 nucleotides (clone 7), respectively. As the deduced amino acid sequences did not represent that of the Sec1 protein (Aalto *et al.,* 1991) the new genes were named *SSO1* and *SSO2* (Suppressor of Sec1 One). The *SSO1* and *SSO2* coding sequences and the deduced amino acid sequences are given in SEQ ID NO: 1 and SEQ ID NO: 3, respectively. The plasmids carrying the *SSO1* and *SSO2* genes were named YEpSSO1 and YEpSSO2, respectively and are shown in Figs. 1A and 1B.

Example 2: Overexpression of the Sso2 protein in yeast transformed with YEpSSO2.

The yeast strain sf750-14D transformed with the control plasmid pMA56 (A) (Ammerer, 1983) or with YEpSSO2 (B) were grown in synthetic complete medium (Sherman *et al.* 1983) lacking Trp. Yeast cell lysates were prepared in the presence of SDS as described by Keränen (1986). Ten μg of total yeast protein present in the lysates were separated by SDS-PAGE and analyzed by Western blotting using polyclonal antibodies made in rabbit against the Sso2 protein and alkaline phosphatase conjugated goat anti-rabbit IgG for detection. As shown in Fig. 2, greatly increased amount of Sso2 protein was seen in the YEpSSO2 transformant.

Example 3: Enhanced production of secreted heterologous protein, *Bacillus* α-amylase in yeast strain sf750-14D overexpressing either *SSO1* or *SSO2*.

The yeast strain sf750-14Dα harboring either *SSO1* or *SSO2* gene on the multicopy plasmids YEpSSO1 or YEpSSO2, respectively, were transformed with a multicopy plasmid YEpαa5 containing *Bacillus* α-amylase gene ligated between the *ADH1* promoter and terminator (Ruohonen *et al.,* 1987), modified for more efficient expression by deleting predicted inhibitory sequences 5' to the promoter element (Ruohonen *et al.,* 1991; Ruohonen *et al.,* manuscript in preparation, a). The yeast strains obtained containing YEpSSO1 and YEpαa5 (VTT-C-92072) or YEpSSO2

and YEpαa5 (VTT-C-92073) were grown in selective medium at 24°C and secretion of α-amylase into the culture medium was monitored by measuring the α-amylase activity using the Phadebas amylase test (Pharmacia Diagnostics AB, Sweden). These strains VTT-C-92072 and VTT-C-92073 were deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) on 30 September 1992 with the accession numbers DSM 7253 and 7254, respectively. As shown in Fig. 3, increased α-amylase activity was obtained in strains which carried either *SSO1* (▲) or *SSO2* (■) on the multicopy plasmid compared with the untransformed control strain (●). Segregation of YEpSSO1 (Δ) or YEpSSO2 (□) off from the transformants reduced the α-amylase secretion to the control level proving that the increased secretion is due to the presence of the *SSO* gene containing plasmids in the transformants. Increased amount of α-amylase protein in the culture medium was detected by Western blotting (Fig. 4). Symbols as for Fig. 3., S = standard (*Bacillus* α-amylase).

Example 4: Enhanced production of secreted foreign protein, *Bacillus* α-amylase and an endogenous protein, invertase in yeast strain DBY746 overexpressing *SSO2*.

The *S. cerevisiae* strain DBY746 (*α his3Δ1 leu2-3 leu2-112 ura3-52 trp1-289 cgh*^{R}) (obtained from David Botstein, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA) harboring the plasmid YEpαa6 containing *Bacillus* α-amylase gene ligated between the *ADH1* promoter and terminator (Ruohonen *et al.*, 1987), modified for more efficient expression by deleting predicted inhibitory sequences 5' to the promoter element (Ruohonen *et al.*, 1991; Ruohonen *et al.*, manuscript in preparation, a) was transformed either with YEpSSO2 or with the control plasmid pMA56 (Ammerer, 1983). The transformants were grown in selective medium at 30°C and secretion of α-amylase into the culture medium was monitored by measuring the α-amylase activity using the Phadebas amylase test (Pharmacia Diagnostics AB, Sweden). As shown in Fig. 5, increased α-amylase activity was obtained in the strain which carried *SSO2* (Δ) on the multicopy plasmid compared with the control strain transformed with the control plasmid without *SSO* gene (○).

No difference was observed in the yeast growth between the control transformant (●) and *SSO2* transformant (▲). Overexpression of *SSO1* increased the secretion of α–amylase in a similar manner. Secretion of the endogenous protein, invertase, was also enhanced under these conditions measured at late logarithmic to early stationary growth phase. The secreted invertase activity in the YEpSSO2 transformant was 1.4 times that in the control transformant containing pMA56. As the enhancing effect of *SSO* overexpression on α–amylase secretion is more pronounced later during the growth, also the invertase secretion should be more enhanced at later time points.

Removal of the predicted inhibitory sequences on the *ADH1* promoter (see above) used for expression of the *SSO2* in YEpSSO2 resulted in prolonged expression of *SSO2* and prolonged existence of increased level of the Sso2 protein and consequently even higher final levels of the *Bacillus* α–amylase secreted into the medium. Expression of *SSO2* on a single copy plasmid from this modified *ADH1* promoter also resulted in increased levels of the Sso2 protein and enhanced secretion of α–amylase.

Example 5: Enhanced production of secreted foreign protein, *Bacillus* α–amylase in yeast overexpressing *SEC1* in combination with normal or increased levels of functional Sso proteins.

The *S. cerevisiae* strain DBY746 harboring the plasmid YEpαa6 containing *Bacillus* α–amylase gene ligated between the *ADH1* promoter and terminator (Ruohonen *et al.*, 1987), modified for more efficient expression by deleting predicted inhibitory sequences 5' to the promoter element (Ruohonen *et al.*, 1991; Ruohonen *et al.*, manuscript in preparation, a) was transformed either with a multicopy plasmid YEpSEC1 expressing the *SEC1* gene or with the control plasmid YEp24H (Aalto *et al.*, 1991; Ruohonen *et al.*, manuscript in preparation, b). The transformants were grown in selective medium at 30°C and secretion of α–amylase into the culture medium was monitored by measuring the α–amylase activity using the Phadebas amylase test (Pharmacia Diagnostics AB, Sweden). As shown in Fig. 6, increased α–amylase activity was obtained in the strains which carried *SEC1* on a multicopy

plasmid (□) compared with the strains transformed with the vector without *SEC1* gene (○). No difference was observed in the growth between the transformants.

Overexpression of both Sec1p and Sso2p at the same time enhanced α-amylase secretion even further. The plasmids expressing the *SSO* genes are available at VTT, Biotechnical Laboratory, Espoo, Finland.

Example 6: Isolation of the *Trichoderma sso* genes by expression in yeast and their expression in *Trichoderma*

A yeast expression gene bank prepared from the *T. reesei* strain QM9414 as described (Buchert *et al.*, FI Pat Appl. 922373) was transformed into the *Saccharomyces cerevisiae* strain H458 (Aalto *et al.*, 1993) (α *SUC2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura 3-1 sso1-δ1::URA3 sso2-δ2::leu2::(GAL1:sso1,HIS3)*) by selecting for Ura-prototrophy on a galactose medium The transformants were transferred onto glucose medium and the plasmid was rescued from the growing colonies and retransformed into the above mentioned strain to verify the complementation. A clone was obtained showing capability to rescue depletion of the Sso proteins on glucose medium and the corresponding plasmid was named pMS51. The *S. cerevisiae* strain obtained, carrying the plasmid pMS51 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) on 5 October 1993 with the accession number DSM 8604. The chromosomal copy of the gene is isolated from a genomic cosmid library (Mäntylä *et al.*, 1992) by using the 5' end of the cDNA clones as a probe, prepared by PCR. The cosmid is isolated from the clones giving a signal, and those corresponding to the above mentioned cDNA are transformed into a *T. reesei* (Penttilä *et al.*, 1987) strain producing CBHI-Fab molecules VTT-D-91418 (CBS 287.91) described in Nyyssönen *et al.*, (Pat. Appl.). Production of CBHI-Fab is studied from the extracellular medium on Solca-floc medium (according to Nyyssönen *et al.*, Pat. Appl.).

Example 7: Isolation of fungal *sso* genes by heterologous hybridization

Genomic DNA from the fungal species *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Pichia stipitis*, *Aspergillus nidulans* and *Trichoderma reesei* were isolated, digested with the *Hin*dIII restriction enzyme, separated electrophoretically in an 0.8% agarose gel and blotted on a nylon filter. Southern hybridization of the filter was carried out at different stringencies using the yeast *SSO1* gene coding region as a probe. Hybridization in a mixture containing 30 % formamide, 6xSSC, 10 x Denhardt's, 0.5% SDS, 100 µg/ml herring sperm DNA and 10 µg/ml polyA at 35 °C and washing 2 x 30 minutes in 2xSSC, 0.1% SDS at 42 °C revealed several hybridizing bands in DNA derived from *S. cerevisiae*, *K. lactis*, *P. stipitis* and *T. reesei* (Fig. 8). When hybridization was performed in less stringent conditions, hybridization was observrd also with *S. pombe* DNA. A genomic *T. reesei* gene library constructed in the λEMBL3 (Frischauf *et al.*, 1983) vector was hybridized by the procedure described above. Clones giving hybridization signals were purified and their hybridizing regions were mapped by digestions and Southern hybridizations of their DNA. The three hybridizing λ clones were designated TSSOa, TSSOb and TSSOc. These clones were deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) on 5 October 1993 with the accession numbers DSM 8601, DSM 8602 and DSM 8603, respectively.

Deposited microorganisms

The following microorganisms were deposited according to the Budapest Treaty at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Mascheroder Weg 1b, D-3300 Braunschweig, Germany.

Strain	Deposition number	Deposition date
Saccharomyces cerevisiae VTT-C-92072 carrying the plasmid YEpSSO1	DSM 7253	30 September 1992
Saccharomyces cerevisiae VTT-C-92073 carrying the plasmid YEpSSO2	DSM 7254	30 September 1992
Saccharomyces cerevisiae H458 (VTT-C-93002) carrying the plasmid pMS51	DSM 8604	5 October 1993
Bacteriophage λ strain TSSOa (VTT-H-93001)	DSM 8601	5 October 1993
Bacteriophage λ strain TSSOb (VTT-H-93002)	DSM 8602	5 October 1993
Bacteriophage λ strain TSSOc (VTT-H-93003)	DSM 8603	5 October 1993

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SEQUENCE LISTING

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(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: FI 92 4494
(B) FILING DATE: 06-OCT-1992

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 870 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: *Saccharomyces cerevisiae*
(B) STRAIN: X2180-1B

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..870

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Tyr Glu Leu Asp Glu Gly Ser Ser Ala Ile Gly Ala Glu Gly His Asp
            20                  25                  30

TTC GTG GGC TTC ATG AAT AAG ATC AGT CAA ATC AAT CGC GAT CTC GAT       144
Phe Val Gly Phe Met Asn Lys Ile Ser Gln Ile Asn Arg Asp Leu Asp
        35                  40                  45

AAG TAC GAC CAT ACC ATC AAC CAG GTC GAT TCT TTG CAT AAG AGG CTA       192
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 65                  70                  75                  80

GAC AAC TTC GTC GCA CAA GCC ACG GAC TTG CAG TTC AAA CTG AAA AAT          288
Asp Asn Phe Val Ala Gln Ala Thr Asp Leu Gln Phe Lys Leu Lys Asn
                 85                  90                  95

GAG ATT AAA AGT GCC CAA AGG GAT GGG ATA CAT GAC ACC AAC AAG CAA          336
Glu Ile Lys Ser Ala Gln Arg Asp Gly Ile His Asp Thr Asn Lys Gln
            100                 105                 110

GCT CAG GCG GAA AAC TCC AGA CAA AGA TTT TTG AAG CTT ATC CAG GAC          384
Ala Gln Ala Glu Asn Ser Arg Gln Arg Phe Leu Lys Leu Ile Gln Asp
        115                 120                 125

TAC AGA ATT GTG GAT TCC AAC TAC AAG GAG GAG AAT AAA GAG CAA GCC          432
Tyr Arg Ile Val Asp Ser Asn Tyr Lys Glu Glu Asn Lys Glu Gln Ala
    130                 135                 140

AAG AGG CAG TAT ATG ATC ATT CAA CCA GAG GCC ACC GAA GAT GAA GTT          480
Lys Arg Gln Tyr Met Ile Ile Gln Pro Glu Ala Thr Glu Asp Glu Val
145                 150                 155                 160

GAA GCA GCC ATA AGC GAT GTA GGG GGC CAG CAG ATC TTC TCA CAA GCA          528
Glu Ala Ala Ile Ser Asp Val Gly Gly Gln Gln Ile Phe Ser Gln Ala
                165                 170                 175

TTG TTG AAT GCT AAC AGA CGT GGG GAA GCC AAG ACT GCT CTT GCG GAA          576
Leu Leu Asn Ala Asn Arg Arg Gly Glu Ala Lys Thr Ala Leu Ala Glu
            180                 185                 190

GTC CAG GCA AGG CAC CAA GAG TTA TTG AAA CTA GAA AAA TCC ATG GCA          624
Val Gln Ala Arg His Gln Glu Leu Leu Lys Leu Glu Lys Ser Met Ala
        195                 200                 205

GAA CTT ACT CAA TTG TTT AAT GAC ATG GAA GAA CTG GTA ATA GAA CAA          672
Glu Leu Thr Gln Leu Phe Asn Asp Met Glu Glu Leu Val Ile Glu Gln
    210                 215                 220

CAA GAA AAC GTA GAC GTC ATC GAC AAG AAC GTT GAA GAC GCT CAA CTC          720
Gln Glu Asn Val Asp Val Ile Asp Lys Asn Val Glu Asp Ala Gln Leu
225                 230                 235                 240

GAC GTA GAA CAG GGT GTC GGT CAT ACC GAT AAA GCC GTC AAG AGT GCC          768
Asp Val Glu Gln Gly Val Gly His Thr Asp Lys Ala Val Lys Ser Ala
                245                 250                 255

AGA AAA GCA AGA AAG AAC AAG ATT AGA TGT TGG TTG ATT GTA TTC GCC          816
Arg Lys Ala Arg Lys Asn Lys Ile Arg Cys Trp Leu Ile Val Phe Ala
            260                 265                 270

ATC ATT GTA GTC GTT GTT GTT GTC GTT GTT GTC CCA GCC GTT GTC AAA          864
Ile Ile Val Val Val Val Val Val Val Val Val Pro Ala Val Val Lys
        275                 280                 285

ACG CGT                                                                 870
Thr Arg
    290
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 290 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ser Tyr Asn Asn Pro Tyr Gln Leu Glu Thr Pro Phe Glu Glu Ser
1 5 10 15

Tyr Glu Leu Asp Glu Gly Ser Ser Ala Ile Gly Ala Glu Gly His Asp
20 25 30

Phe Val Gly Phe Met Asn Lys Ile Ser Gln Ile Asn Arg Asp Leu Asp
35 40 45

Lys Tyr Asp His Thr Ile Asn Gln Val Asp Ser Leu His Lys Arg Leu
50 55 60

Leu Thr Glu Val Asn Glu Glu Gln Ala Ser His Leu Arg His Ser Leu
65 70 75 80

Asp Asn Phe Val Ala Gln Ala Thr Asp Leu Gln Phe Lys Leu Lys Asn
85 90 95

Glu Ile Lys Ser Ala Gln Arg Asp Gly Ile His Asp Thr Asn Lys Gln
100 105 110

Ala Gln Ala Glu Asn Ser Arg Gln Arg Phe Leu Lys Leu Ile Gln Asp
115 120 125

Tyr Arg Ile Val Asp Ser Asn Tyr Lys Glu Glu Asn Lys Glu Gln Ala
130 135 140

Lys Arg Gln Tyr Met Ile Ile Gln Pro Glu Ala Thr Glu Asp Glu Val
145 150 155 160

Glu Ala Ala Ile Ser Asp Val Gly Gly Gln Gln Ile Phe Ser Gln Ala
165 170 175

Leu Leu Asn Ala Asn Arg Arg Gly Glu Ala Lys Thr Ala Leu Ala Glu
180 185 190

Val Gln Ala Arg His Gln Glu Leu Leu Lys Leu Glu Lys Ser Met Ala
195 200 205

Glu Leu Thr Gln Leu Phe Asn Asp Met Glu Glu Leu Val Ile Glu Gln
210 215 220

Gln Glu Asn Val Asp Val Ile Asp Lys Asn Val Glu Asp Ala Gln Leu
225 230 235 240

Asp Val Glu Gln Gly Val Gly His Thr Asp Lys Ala Val Lys Ser Ala
245 250 255

Arg Lys Ala Arg Lys Asn Lys Ile Arg Cys Trp Leu Ile Val Phe Ala
260 265 270

Ile Ile Val Val Val Val Val Val Val Val Pro Ala Val Val Lys
275 280 285

Thr Arg
290

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 885 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: *Saccharomyces cerevisiae*
(B) STRAIN: X2180-1B

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..885

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
ATG AGC AAC GCT AAT CCT TAT GAG AAT AAC AAT CCG TAC GCT GAA AAC          48
Met Ser Asn Ala Asn Pro Tyr Glu Asn Asn Asn Pro Tyr Ala Glu Asn
 1               5                  10                  15

TAT GAA ATG CAA GAG GAC TTG AAC AAT GCT CCT ACT GGT CAC TCA GAT          96
Tyr Glu Met Gln Glu Asp Leu Asn Asn Ala Pro Thr Gly His Ser Asp
            20                  25                  30

GGT AGC GAC GAT TTC GTA GCT TTT ATG AAC AAG ATC AAC TCA ATA AAT         144
Gly Ser Asp Asp Phe Val Ala Phe Met Asn Lys Ile Asn Ser Ile Asn
        35                  40                  45

GCT AAC TTG TCC AGG TAC GAA AAC ATT ATC AAC CAA ATT GAT GCG CAA         192
Ala Asn Leu Ser Arg Tyr Glu Asn Ile Ile Asn Gln Ile Asp Ala Gln
    50                  55                  60

CAC AAA GAC CTA CTT ACT CAA GTG AGT GAG GAA CAG GAG ATG GAA TTG         240
His Lys Asp Leu Leu Thr Gln Val Ser Glu Glu Gln Glu Met Glu Leu
65                  70                  75                  80

AGA CGT TCT TTG GAC GAT TAC ATC TCT CAG GCC ACA GAT TTG CAG TAT         288
Arg Arg Ser Leu Asp Asp Tyr Ile Ser Gln Ala Thr Asp Leu Gln Tyr
                85                  90                  95

CAA TTG AAA GCG GAT ATC AAA GAT GCC CAG AGA GAC GGA TTG CAC GAC         336
Gln Leu Lys Ala Asp Ile Lys Asp Ala Gln Arg Asp Gly Leu His Asp
            100                 105                 110

TCT AAT AAA CAG GCA CAA GCT GAA AAT TGC AGA CAG AAA TTC TTA AAA         384
Ser Asn Lys Gln Ala Gln Ala Glu Asn Cys Arg Gln Lys Phe Leu Lys
        115                 120                 125

TTA ATT CAA GAC TAC AGA ATT ATC GAT TCT AAC TAC AAA GAA GAA AGC         432
Leu Ile Gln Asp Tyr Arg Ile Ile Asp Ser Asn Tyr Lys Glu Glu Ser
    130                 135                 140

AAA GAG CAG GCG AAG AGA CAG TAC ACA ATT ATC CAA CCG GAA GCC ACT         480
Lys Glu Gln Ala Lys Arg Gln Tyr Thr Ile Ile Gln Pro Glu Ala Thr
145                 150                 155                 160

GAC GAA GAA GTG GAA GCC GCC ATC AAC GAT GTC AAT GGC CAG CAG ATC         528
Asp Glu Glu Val Glu Ala Ala Ile Asn Asp Val Asn Gly Gln Gln Ile
                165                 170                 175
```

```
TTT TCC CAA GCG TTG CTA AAC GCC AAT AGA CGT GGT GAG GCC AAG ACA          576
Phe Ser Gln Ala Leu Leu Asn Ala Asn Arg Arg Gly Glu Ala Lys Thr
            180                 185                 190

GCA TTG GCC GAA GTA CAG GCT AGA CAT CAA GAG TTG TTG AAG TTG GAA          624
Ala Leu Ala Glu Val Gln Ala Arg His Gln Glu Leu Leu Lys Leu Glu
        195                 200                 205

AAA ACA ATG GCT GAA CTT ACC CAA TTG TTC AAT GAC ATG AAA GAG TTG          672
Lys Thr Met Ala Glu Leu Thr Gln Leu Phe Asn Asp Met Lys Glu Leu
    210                 215                 220

GTC ATC GAA CAA CAA GAA AAT GTG GAT GTC ATT GAC AAA AAC GTC GAA          720
Val Ile Glu Gln Gln Glu Asn Val Asp Val Ile Asp Lys Asn Val Glu
225                 230                 235                 240

GAC GCT CAG CAA GAT GTA GAG CAA GGT GTG GGT CAC ACC AAC AAG GCC          768
Asp Ala Gln Gln Asp Val Glu Gln Gly Val Gly His Thr Asn Lys Ala
                245                 250                 255

GTT AAG AGT GCC AGA AAA GCA AGA AAA AAC AAA ATA AGA TGT TTG ATC          816
Val Lys Ser Ala Arg Lys Ala Arg Lys Asn Lys Ile Arg Cys Leu Ile
            260                 265                 270

ATC TGC TTT ATT ATC TTT GCT ATT GTT GTT GTC GTT GTG GTT GTT CCA          864
Ile Cys Phe Ile Ile Phe Ala Ile Val Val Val Val Val Val Val Pro
        275                 280                 285

TCC GTT GTG GAA ACA AGA AAG                                             885
Ser Val Val Glu Thr Arg Lys
    290                 295
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 295 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Ser Asn Ala Asn Pro Tyr Glu Asn Asn Asn Pro Tyr Ala Glu Asn
  1               5                  10                  15

Tyr Glu Met Gln Glu Asp Leu Asn Asn Ala Pro Thr Gly His Ser Asp
            20                  25                  30

Gly Ser Asp Asp Phe Val Ala Phe Met Asn Lys Ile Asn Ser Ile Asn
        35                  40                  45

Ala Asn Leu Ser Arg Tyr Glu Asn Ile Ile Asn Gln Ile Asp Ala Gln
    50                  55                  60

His Lys Asp Leu Leu Thr Gln Val Ser Glu Glu Gln Glu Met Glu Leu
65                  70                  75                  80

Arg Arg Ser Leu Asp Asp Tyr Ile Ser Gln Ala Thr Asp Leu Gln Tyr
                85                  90                  95

Gln Leu Lys Ala Asp Ile Lys Asp Ala Gln Arg Asp Gly Leu His Asp
            100                 105                 110
```

Ser Asn Lys Gln Ala Gln Ala Glu Asn Cys Arg Gln Lys Phe Leu Lys
115 120 125

Leu Ile Gln Asp Tyr Arg Ile Ile Asp Ser Asn Tyr Lys Glu Glu Ser
130 135 140

Lys Glu Gln Ala Lys Arg Gln Tyr Thr Ile Ile Gln Pro Glu Ala Thr
145 150 155 160

Asp Glu Glu Val Glu Ala Ala Ile Asn Asp Val Asn Gly Gln Gln Ile
165 170 175

Phe Ser Gln Ala Leu Leu Asn Ala Asn Arg Arg Gly Glu Ala Lys Thr
180 185 190

Ala Leu Ala Glu Val Gln Ala Arg His Gln Glu Leu Leu Lys Leu Glu
195 200 205

Lys Thr Met Ala Glu Leu Thr Gln Leu Phe Asn Asp Met Lys Glu Leu
210 215 220

Val Ile Glu Gln Gln Glu Asn Val Asp Val Ile Asp Lys Asn Val Glu
225 230 235 240

Asp Ala Gln Gln Asp Val Glu Gln Gly Val Gly His Thr Asn Lys Ala
245 250 255

Val Lys Ser Ala Arg Lys Ala Arg Lys Asn Lys Ile Arg Cys Leu Ile
260 265 270

Ile Cys Phe Ile Ile Phe Ala Ile Val Val Val Val Val Val Val Pro
275 280 285

Ser Val Val Glu Thr Arg Lys
290 295

CLAIMS

WE CLAIM:

1. An isolated DNA sequence of a *sec1* suppressor gene *SSO* selected from the group consisting of:

SSO1 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:2,

SSO2 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:4, and

DNA sequences which hybridize with a DNA having a nucleotide sequence depicted in SEQ ID NO: 1 or 3 in a mixture containing 30% formamide, 6xSSC, 10 x Denhardt's, 0.5% SDS, 100 µg/ml herring sperm DNA, and 10 µg/ml polyA at 35°C and washing 2 x 30 minutes in 2xSSC, 0.1% SDS at 42°C.

2. A vector comprising a DNA sequence according to claim 1.

3. The vector according to claim 2, wherein said vector is a filamentous fungus expression vector.

4. The vector according to claim 3, wherein expression of DNA in said vector is under control of a regulatory region selected from the group consisting of the *sso* promoter, the *cbh1* promoter, the *cbh2* promoter, the *egl1* promoter, the *egl2* promoter, the *tef1* promoter, the *pgk* promoter, the *gpd* promoter, the *pki* promoter, the glucoamylase promoter, the α-amylase promoter, and the alcohol dehydrogenase promoter.

5. The vector according to claim 2, wherein said vector autonomously replicates in a fungal cell.

6. The vector according to claim 2, wherein said vector integrates into the chromosome of a fungal cell.

7. The vector according to claim 2, wherein said vector is a yeast expression vector wherein gene expression is controlled by yeast regulatory regions.

8. The vector according to claim 7, wherein said yeast regulatory regions are selected from the group consisting of the promoter sequence of *SSO1*, the promoter sequence of *SSO2*, the promoter sequence of *SEC1*, the promoter sequence of *GAL1*, the promoter sequence of *GAL10*, the promoter sequence of *ADH1*, and the promoter sequence of asparagine synthetase gene.

9. The vector according to claim 2, wherein said vector is a fungal vector selected from the group consisting of YEpSSO1 and YEpSSO2.

10. A recombinant fungal host cell comprising a DNA sequence according to claim 1.

11. The recombinant fungal host cell according to claim 10 selected from the group consisting of *Saccharomyces* spp., *Trichoderma* spp., *Kluyveromyces* spp., *Schizosaccharomyces pombe*, *Pichia* spp., *Hansenula* spp., *Yarrowia* spp., *Aspergillus* spp., and *Neurospora* spp.

12. The recombinant fungal host cell according to claim 10 selected from the group consisting of *Saccharomyces cerevisiae*, strain VTT-C-92072 (DSM 7253) and *Saccharomyces cerevisiae*, strain VTT-C-92073 (DSM 7254).

13. A method for enhancing expression of Sso protein in a recombinant fungal host cell, comprising the steps of:

(a) isolating DNA encoding an Sso protein from a suitable donor organism, wherein the Sso protein is selected from the group consisting of:

the polypeptide as depicted in SEQ ID NO:2,

the polypeptide as depicted in SEQ ID NO:4, and

polypeptides encoded by DNA sequences which hybridize with a DNA having a nucleotide sequence depicted in SEQ ID NO: 1 or 3 in a mixture containing 30% formamide, 6xSSC, 10 x Denhardt's, 0.5% SDS, 100 μg/ml herring sperm DNA, and 10 μg/ml polyA at 35°C and washing 2 x 30 minutes in 2xSSC, 0.1% SDS at

42°C;

(b) obtaining a vector comprising said DNA; and

(c) transforming said vector into a suitable fungal host cell wherein Sso protein expression is enhanced.

14. The method according to claim 13, wherein said suitable fungal host cell is selected from the group consisting of *Saccharomyces* spp., *Trichoderma* spp., *Kluyveromyces* spp., *Schizosaccharomyces pombe*, *Pichia* spp., *Hansenula* spp., *Yarrowia* spp., *Aspergillus* spp., and *Neurospora* spp.

15. A method for increasing production of a secreted protein in a fungal host cell, comprising the steps of:

(a) obtaining a vector comprising an isolated DNA encoding said secreted protein;

(b) transforming said vector into a suitable fungal host cell having enhanced expression of an *SSO* gene, which has the DNA sequence selected from the group consisting of:

SSO1 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:2,

SSO2 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:4, and

DNA sequences which hybridize with a DNA having a nucleotide sequence depicted in SEQ ID NO: 1 or 3 in a mixture containing 30% formamide, 6xSSC, 10 x Denhardt's, 0.5% SDS, 100 µg/ml herring sperm DNA, and 10 µg/ml polyA at 35°C and washing 2 x 30 minutes in 2xSSC, 0.1% SDS at 42°C;

(c) culturing said host cell under conditions suitable for expression of said secreted protein; and

(d) purifying said secreted protein from said culture medium.

16. A method for increasing production of a secreted protein in a fungal host cell, comprising the steps of:

(a) obtaining a vector comprising an isolated DNA encoding said secreted protein;

(b) transforming said vector into a suitable fungal host cell having enhanced expression of an *SSO* gene, which has the DNA sequence selected from the group consisting of:

SSO1 DNA sequence encoding a polypeptide comprising an amino acid

sequence as depicted in SEQ ID NO:2,

SSO2 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:4, and

DNA sequences which hybridize with a DNA having a nucleotide sequence depicted in SEQ ID NO: 1 or 3 in a mixture containing 30% formamide, 6xSSC, 10 x Denhardt's, 0.5% SDS, 100 µg/ml herring sperm DNA, and 10 µg/ml polyA at 35°C and washing 2 x 30 minutes in 2xSSC, 0.1% SDS at 42°C,

and a second gene involved in the secretory pathway, said second gene being capable of interacting with said SSO gene;

(c) culturing said host cell in a suitable culture medium; and

(d) purifying said secreted protein from said culture medium.

17. The method according to claim 16, wherein the gene involved in the secretory pathway and capable of interacting with said *SSO* gene is *SEC1* gene.

18. A method for increasing production of an endogenous secreted protein in a fungal host cell, comprising the steps of:

(a) incorporating into a fungal host cell which produces said endogenous secreted protein an *SSO* DNA sequence selected from the group consisting of:

SSO1 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:2,

SSO2 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:4, and

DNA sequences which hybridize with a DNA having a nucleotide sequence depicted in SEQ ID NO: 1 or 3 in a mixture containing 30% formamide, 6xSSC, 10 x Denhardt's, 0.5% SDS, 100 µg/ml herring sperm DNA, and 10 µg/ml polyA at 35°C and washing 2 x 30 minutes in 2xSSC, 0.1% SDS at 42°C;

(b) screening for host cells in which expression of endogenous secreted protein is enhanced;

(c) culturing said host cells in a suitable culture medium; and

(d) purifying said endogenous secreted protein from said culture medium.

19. A method for biomass production in a fungal host cell, comprising the steps of:

(a) obtaining a fungal vector comprising an isolated DNA encoding a hydrolytic enzyme;

(b) transforming said fungal vector into a suitable fungal host expressing enhanced levels of *SSO* genes, which have a DNA sequence selected from the group consisting of:

SSO1 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:2,

SSO2 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:4, and

DNA sequences which hybridize with a DNA having a nucleotide sequence depicted in SEQ ID NO: 1 or 3 in a mixture containing 30% formamide, 6xSSC, 10 x Denhardt's, 0.5% SDS, 100 µg/ml herring sperm DNA, and 10 µg/ml polyA at 35°C and washing 2 x 30 minutes in 2xSSC, 0.1% SDS at 42°C; and

(c) culturing said host cells in a culture medium suitable for biomass production.

20. A method for biomass production in a fungal host cell, comprising the steps of:

(a) obtaining a fungal vector comprising an isolated DNA encoding a hydrolytic enzyme;

(b) transforming said fungal vector into a suitable fungal host cell expressing enhanced levels of *SSO* genes, which have a DNA sequence selected from the group consisting of:

SSO1 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:2,

SSO2 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:4, and

DNA sequences which hybridize with a DNA having a nucleotide sequence depicted in SEQ ID NO: 1 or 3 in a mixture containing 30% formamide, 6xSSC, 10 x Denhardt's, 0.5% SDS, 100 µg/ml herring sperm DNA, and 10 µg/ml polyA at 35°C and washing 2 x 30 minutes in 2xSSC, 0.1% SDS at 42°C,

and genes involved in the secretory pathway being capable of interacting with said *SSO* genes; and

(c) culturing said host cells in a culture medium suitable for biomass production.

21. The method according to claim 20, wherein the gene involved in the secretory pathway and capable of interacting with said *SSO* gene is *SEC1* gene.

22. A method for increasing production of an endogenous secreted protein in a fungal host cell, comprising the steps of:

(a) incorporating into a fungal host cell which produces said endogenous secreted protein an *SSO* gene selected from the group consisting of:

SSO1 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:2,

SSO2 DNA sequence encoding a polypeptide comprising an amino acid sequence as depicted in SEQ ID NO:4, and

DNA sequences which hybridize with a DNA having a nucleotide sequence depicted in SEQ ID NO: 1 or 3 in a mixture containing 30% formamide, 6xSSC, 10 x Denhardt's, 0.5% SDS, 100 µg/ml herring sperm DNA, and 10 µg/ml polyA at 35°C and washing 2 x 30 minutes in 2xSSC, 0.1% SDS at 42°C,

and a second gene involved in the secretory pathway, said second gene being capable of interacting with said *SSO* gene;

(b) selecting host cells in which expression of endogenous secreted protein is enhanced;

(c) culturing said host cells in a suitable culture medium; and

(d) isolating said endogenous secreted protein from said culture medium.

23. The method according to claim 22, wherein the second gene involved in the secretory pathway and capable of interacting with said *SSO* gene is *SEC1* gene.

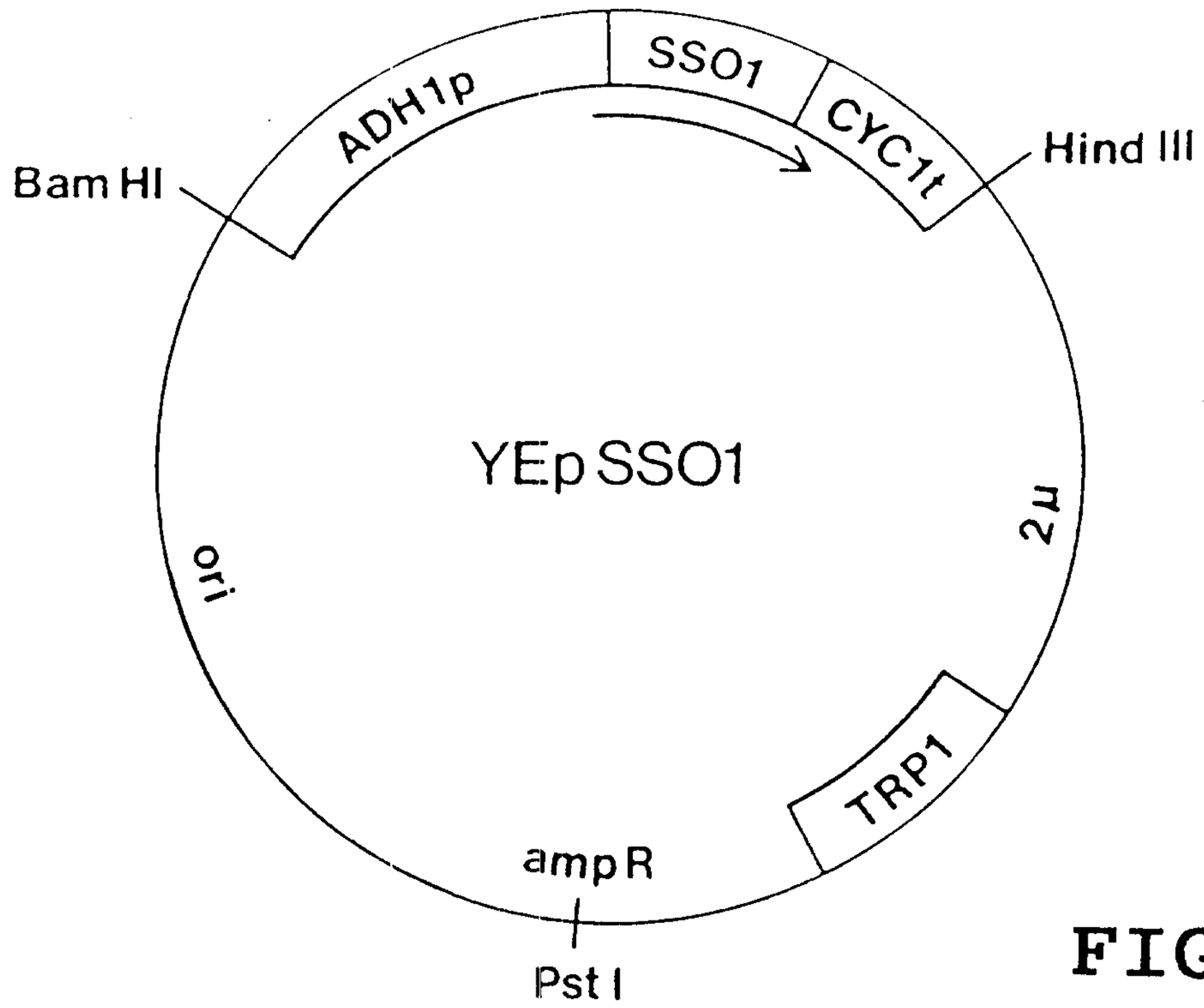

ADH1p
SSO1
CYC1t
Bam HI
Hind III
YEp SSO1
ori
2μ
TRP1
ampR
Pst I

FIG. 1A

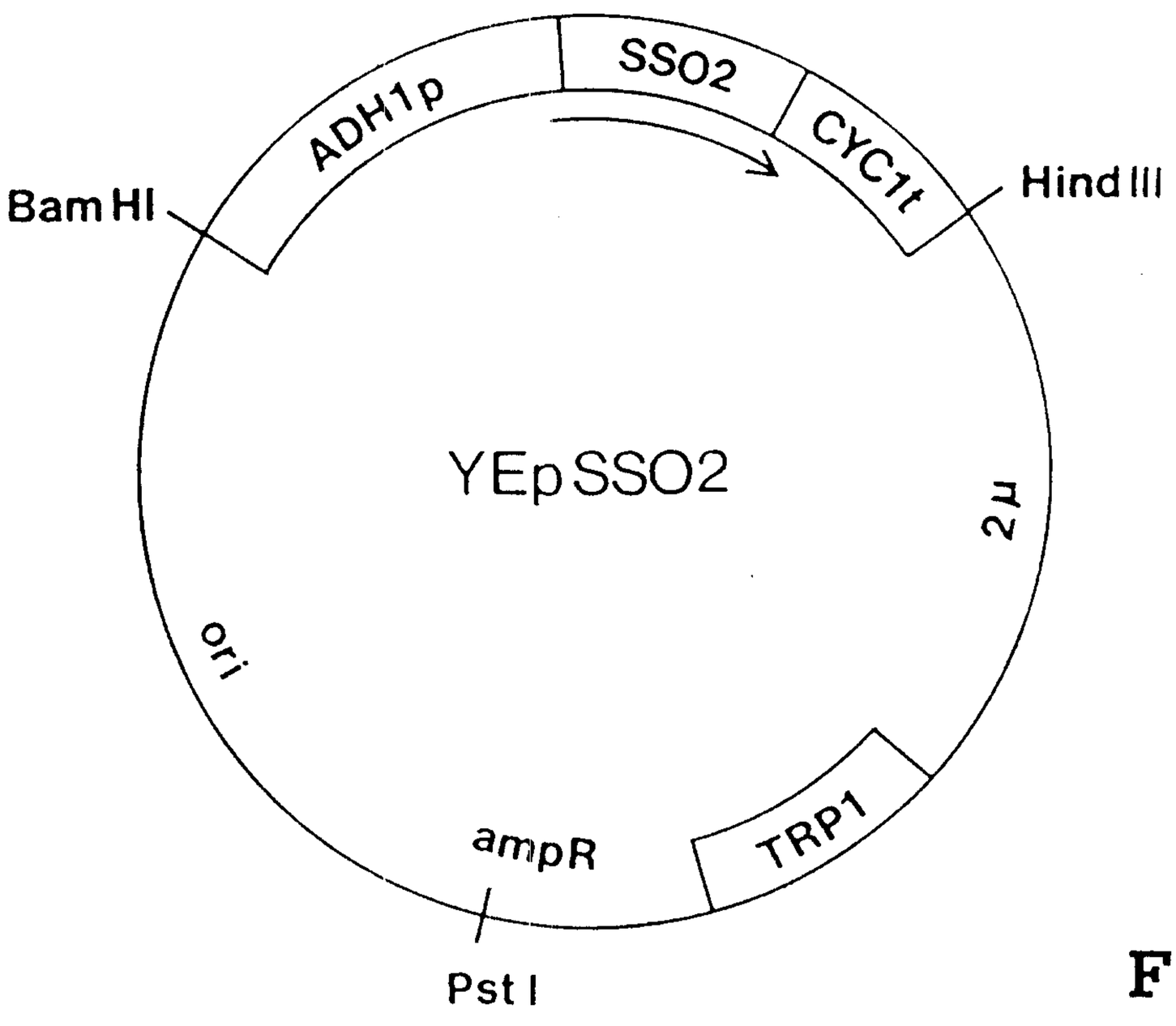

ADH1p
SSO2
CYC1t
Bam HI
Hind III
YEp SSO2
ori
2μ
TRP1
ampR
Pst I

FIG. 1B

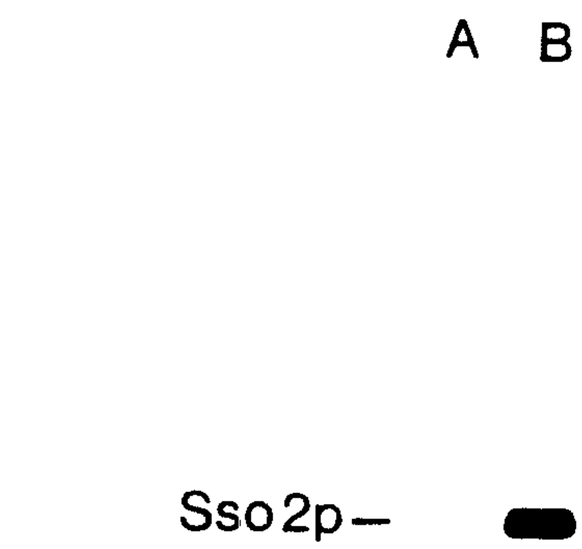


FIG. 2

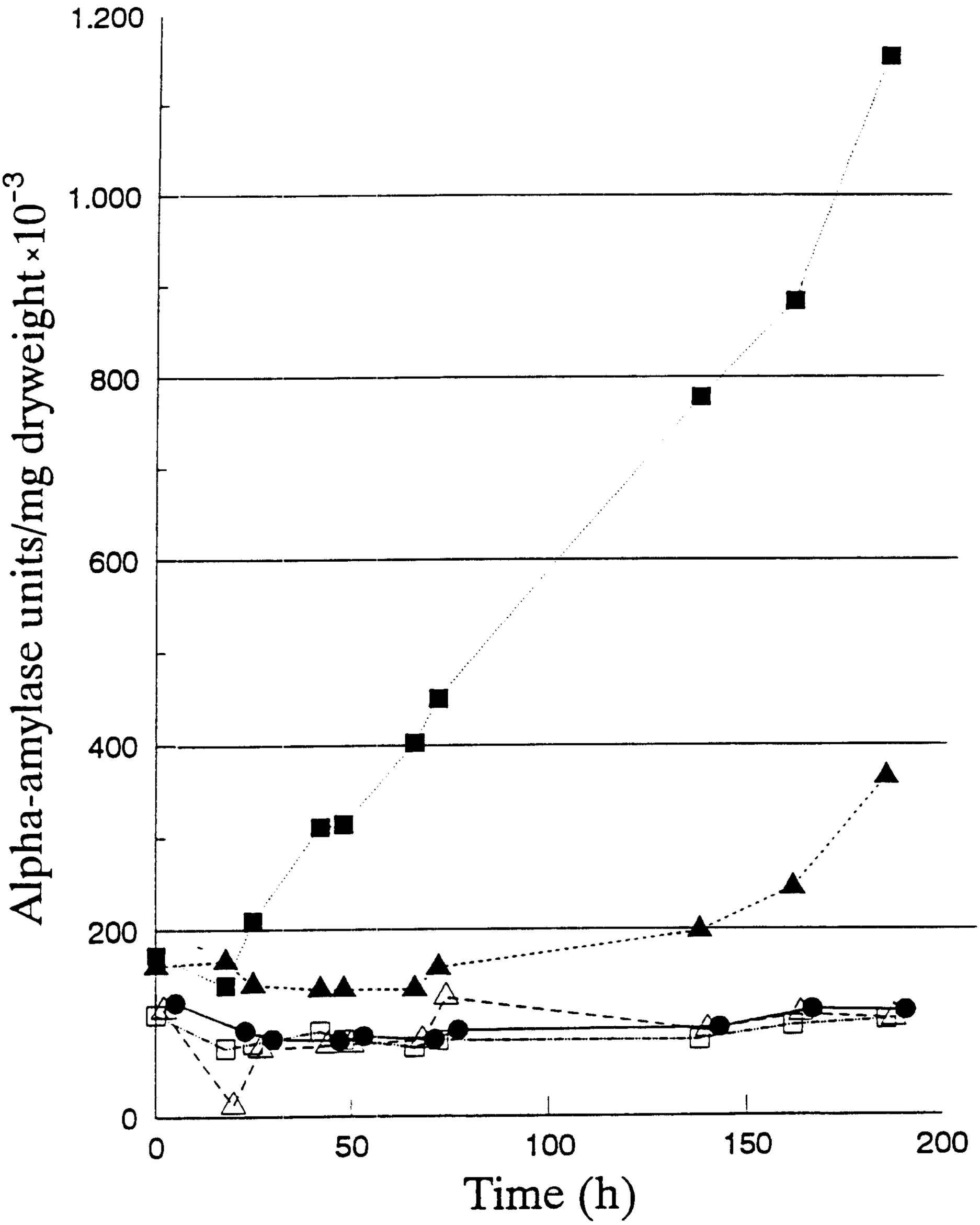

1.200
1.000
800
600
400
200
0
Alpha-amylase units/mg dryweight ×10-3
0
50
100
150
200
Time (h)

FIG. 3

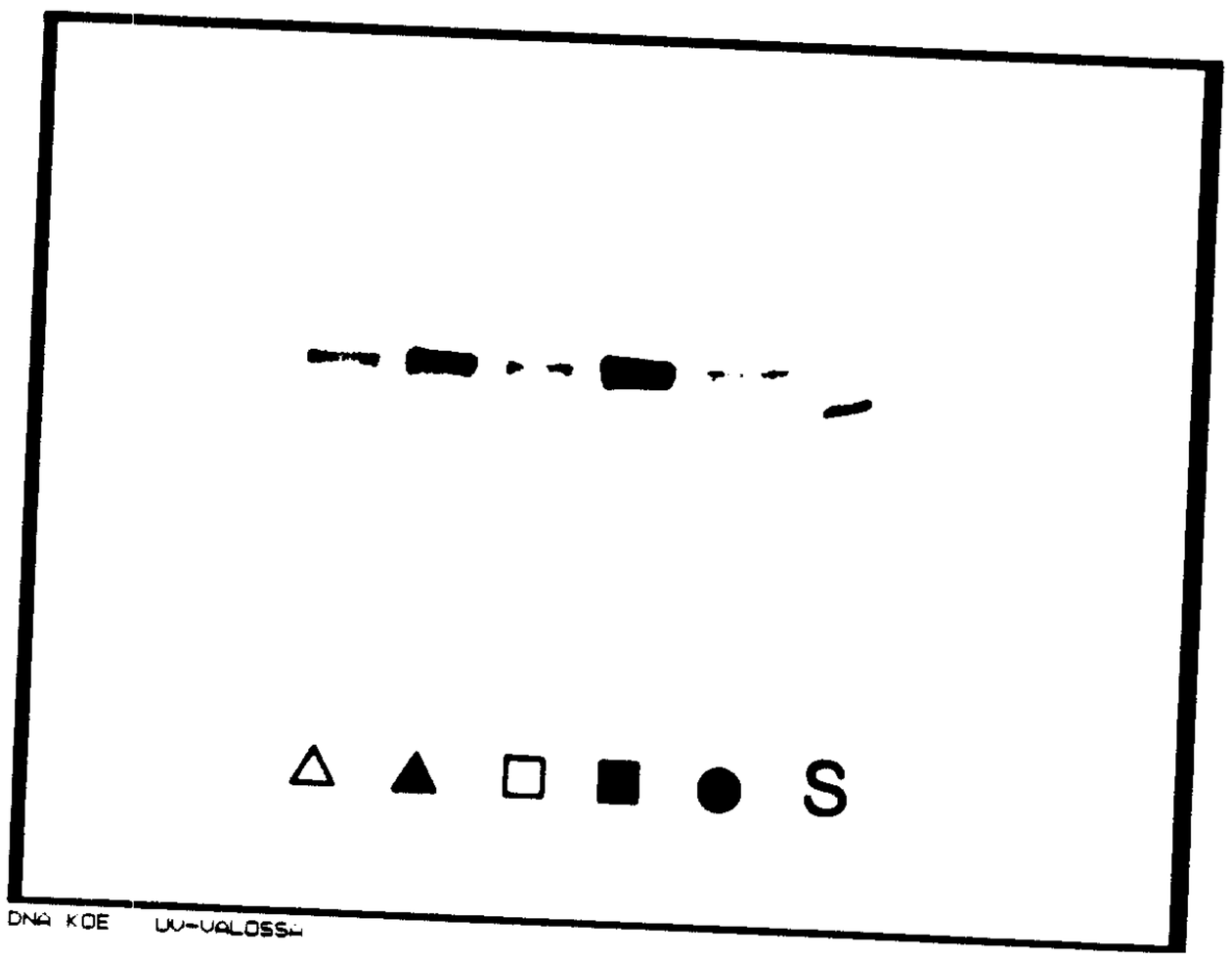


FIG. 4

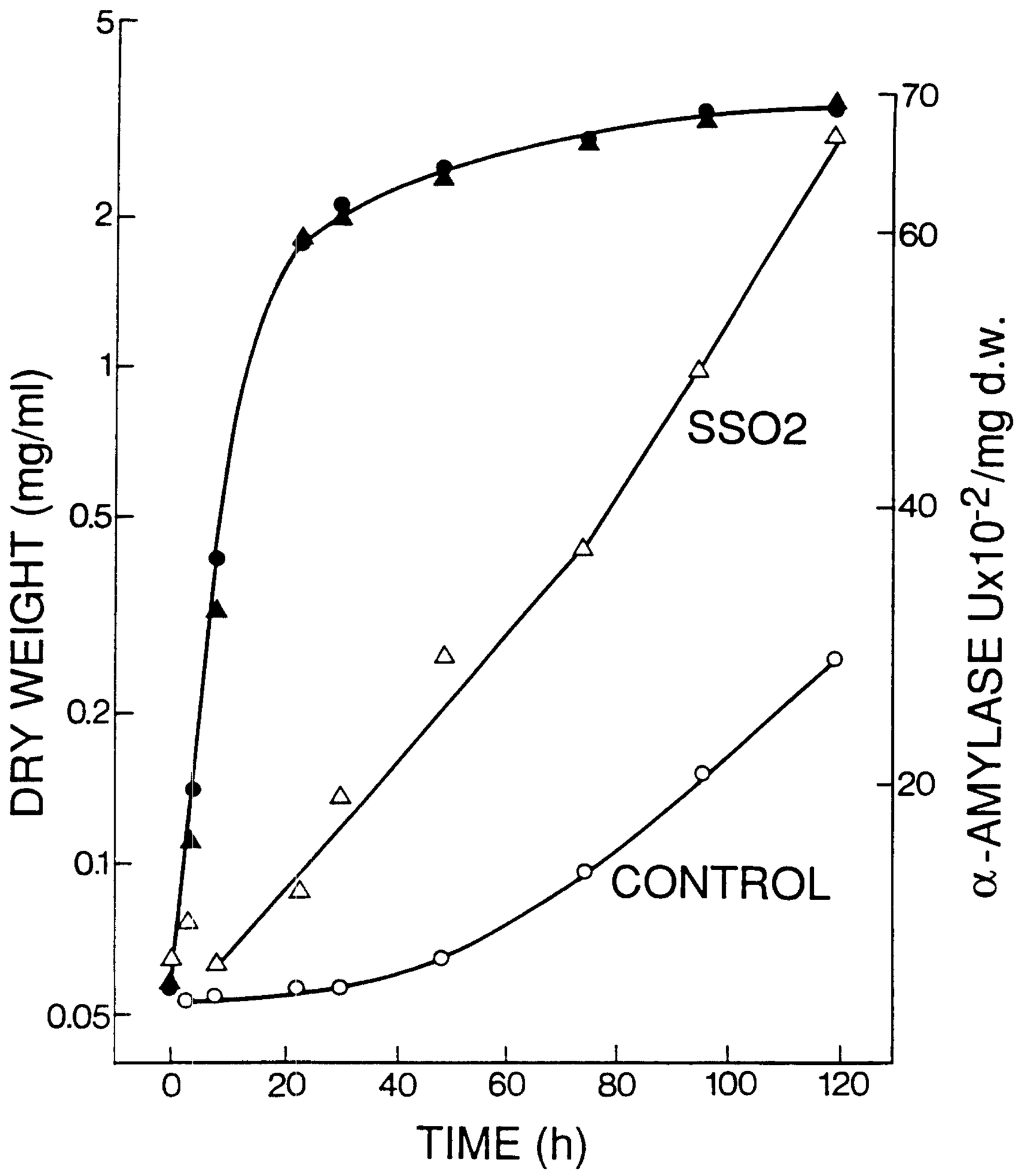

DRY WEIGHT (mg/ml)
α-AMYLASE U x10-2/mg d.w.
TIME (h)
SSO2
CONTROL
5
2
1
0.5
0.2
0.1
0.05
70
60
40
20
0
20
40
60
80
100
120

FIG. 5

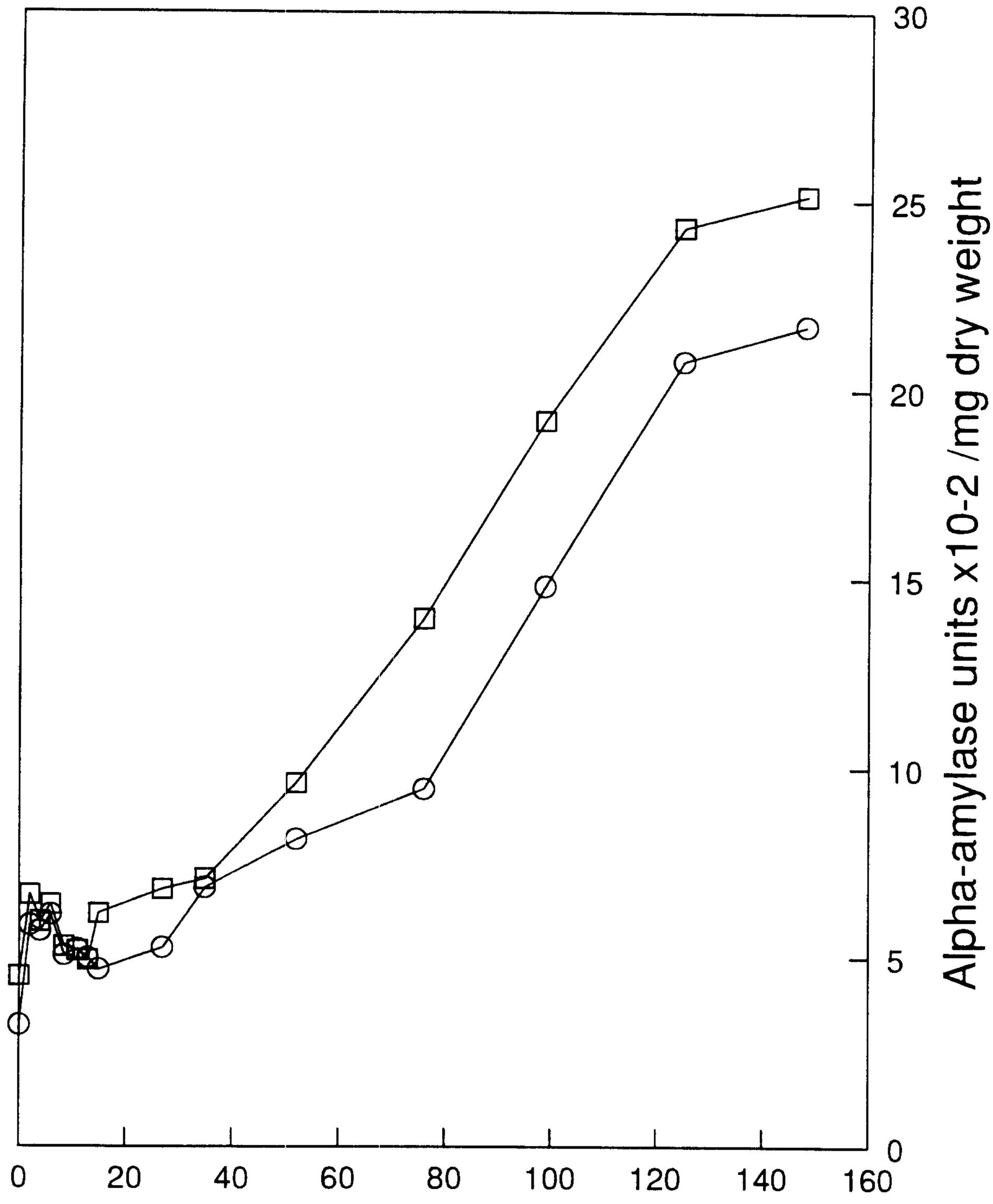

30
25
20
15
10
5
0
Alpha-amylase units x10-2 /mg dry weight
0
20
40
60
80
100
120
140
160
Time (h)
SEC1
control

FIG. 6

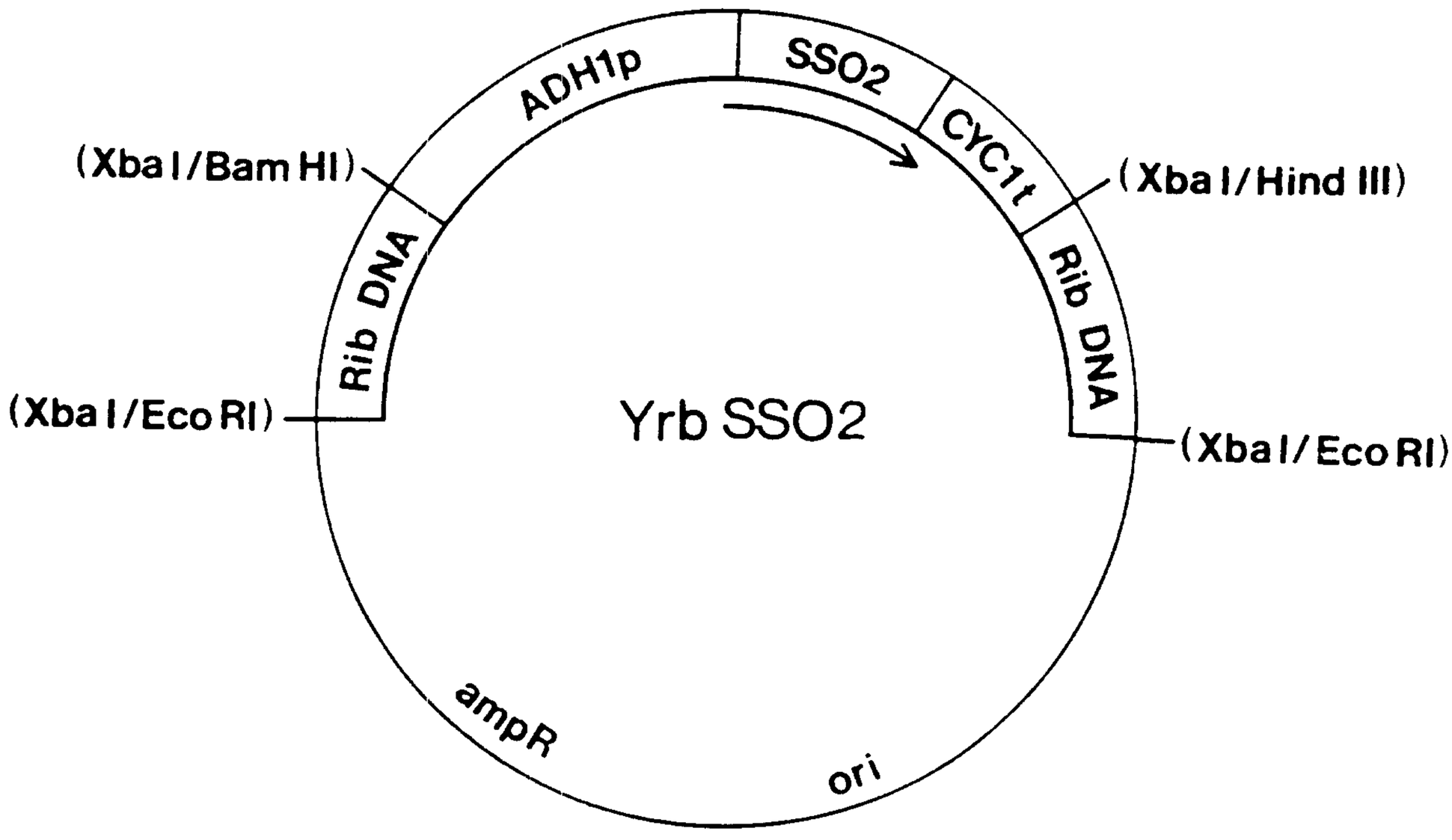
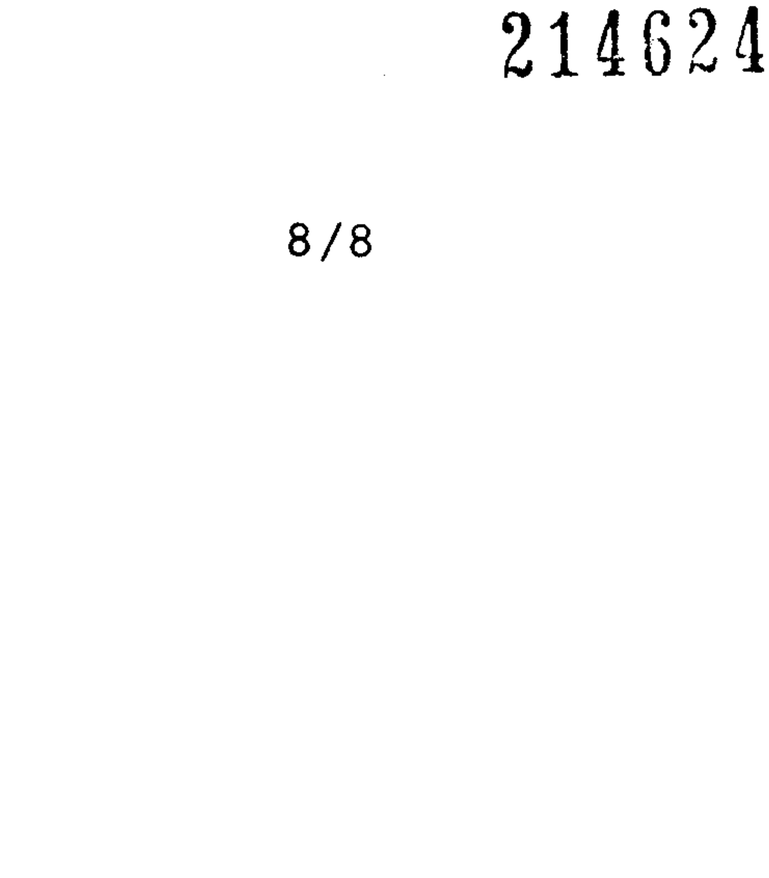

ADH1p
SSO2
CYC1t
Rib DNA
Rib DNA
(Xba I/Bam HI)
(Xba I/Hind III)
(Xba I/Eco RI)
(Xba I/Eco RI)
Yrb SSO2
ampR
ori

FIG. 7



1. *Saccharomyces cerevisiae*
2. *Schizosaccharomyces pombe*
3. *Kluyveromyces lactis*
4. *Pichia stipitis*
5. *Aspergillus nidulans*
6. *Trichoderma reesei*
7. plasmid control

FIG. 8