



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 (71) Applicant (for all designated States except US): VALTION TEKNIILLINEN TUTKIMUSKESKUS [FI/FI]; Vuori- miehentie 5, FIN-02150 Espoo (FI). (72) Inventors; and (75) Inventors/Applicants (for US only) : KERÄNEN, Sirkka [FI/FI]; Rahakamarinkatu 4 B 12, FIN-00240 Helsinki (FI). AALTO, Markku [FI/FI]; Pellonperäntie 4, FIN- 00380 Helsinki (FI). OUTOLA, Mika [FI/FI]; Hatunte- kijänkuja 3-5 B 55, FIN-00750 Helsinki (FI). RONNE, Hans [SE/SE]; Dirigentvägen 169, S-756 45 Uppsala (SE). PENTTILÄ, Merja [FI/FI]; Vähäntuvantie 9 A 6, FIN-00390 Helsinki (FI).		(74) Agent: OY JALO ANT-WUORINEN AB; Iso Roober- tinkatu 4-6 A, FIN-00120 Helsinki (FI). (81) Designated States: AU, CA, FI, JP, NO, NZ, US, Euro- pean patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: INCREASED PRODUCTION OF SECRETED PROTEINS BY RECOMBINANT EUKARYOTIC CELLS (57) Abstract The invention relates to recombinant DNA technology. Specifically this invention relates to new recombinant eukaryotic cells transformed with <i>SSO</i> genes. Eukaryotic cells transformed with several copies of <i>SSO</i> 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.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

INCREASED PRODUCTION OF SECRETED PROTEINS BY RECOMBINANT EUKARYOTIC CELLS

Field of the invention

5

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.

10

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.

15

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

20
25
30

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

5 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
10 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.

15 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.

20 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
25 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.

30 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).

5 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
10 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
15 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.

20 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
25 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
30 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
5 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
10 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.
15 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).

20 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
25 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*.

30 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

Fig. 1 shows 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.

5 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.

10 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.

15 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.

20 **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
25 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
30 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.

5

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.

10

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.

15

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.

20

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*

25

30

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
5 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
10 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, GPY55-15Ba; VTT-A-63015) any
15 *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* spp., *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
20 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
25 (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
30 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. Successful 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.

5 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.

15

20

25

30 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 *ssg* gene(s) need to be expressed from constitutive promoters or
5 promoters functioning on glucose medium.

For filamentous fungi the *ssg* 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 *ssg* gene itself are for instance the other cellulase promoters, *cbh2*,
10 *egl1*, *egl2*, or *tefl*, *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 *ssg* gene integrated into the genome (Penttilä *et al.*, 1987) and from these the strain with optimal level of *ssg* expression for growth and enhanced secretion can be screened.

15 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.*
20 *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*
25 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.

30 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.

5 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
- 10 (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

15 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
- 20 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
- 25 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).

30 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:

- 5 (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)
10 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
- 15 (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:

- 20 (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
- 25 (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
30 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;
- 10 (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
- 15 (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;
- 25 (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
- 30 *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).

5

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.

10 EXPERIMENTAL

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

15 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 sf750-14D α (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
20 McConaughy (1983) from strain X2180-1B on a 2 μ 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
25 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
30 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 Qne). 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 Fig. 1.

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 YEpα5 containing *Bacillus* α-amylase gene ligated between the *ADHI* 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 YEpα5 (VTT-C-92072) or YEpSSO2

and YEp α a5 (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
5 von Mikroorganismen und Zellkulturen GmbH (DSMZ) 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 ($\blacktriangle$) or SSO2 ($\blacksquare$) on the multicopy plasmid compared with the untransformed control strain ($\bullet$). Segregation of YEpSSO1 (Δ) or YEpSSO2 ($\square$) off from the transformants
10 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).

15

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

20 The *S. cerevisiae* strain DBY746 (α *his3 Δ 1 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 YEp α a6 containing *Bacillus* α -amylase gene ligated between the *ADHI* promoter and terminator (Ruohonen *et al.*, 1987), modified for more efficient expression by deleting predicted inhibitory
25 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
30 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 ($\circ$).

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 YEp α a6 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*

10

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- δ 1::URA3 sso2- δ 2::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 (DSMZ) 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 Nyssönen *et al.*, (Pat. Appl.). Production of CBHI-Fab is studied from the extracellular medium on Solca-floc medium (according to Nyssönen *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*
5 *nidulans* and *Trichoderma reesei* were isolated, digested with the *Hind*III 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
10 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 observed also with *S. pombe* DNA. A genomic *T. reesei* gene library constructed in the λEMBL3 (Frischauf *et al.*,
15 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 (DSMZ) on 5
20 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),
5 Mascheroder Weg 1b, D-3300 Braunschweig, Germany.

	Strain	Deposition number	Deposition date
10	<i>Saccharomyces cerevisiae</i> VTT-C-92072 carrying the plasmid YEpSSO1	DSM 7253	30 September 1992
15	<i>Saccharomyces cerevisiae</i> VTT-C-92073 carrying the plasmid YEpSSO2	DSM 7254	30 September 1992
20	<i>Saccharomyces cerevisiae</i> 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
25	Bacteriophage λ strain TSSOb (VTT-H-93002)	DSM 8602	5 October 1993
	Bacteriophage λ strain TSSOc (VTT-H-93003)	DSM 8603	5 October 1993

References

- 5 Aalto, M.K., Keränen, S. and Ronne, H. 1992. A family of proteins involved in intracellular transport. *Cell* 68, 181–182.
- Aalto, M.K., Ronne, H. and Keränen, S. 1993. Yeast syntaxins Sso1p and Sso2p belong to a family of membrane proteins that function in vesicular transport. *EMBO J.* 12, (in press).
- 10 Aalto, M.K., Ruohonen, L., Hosono, K. and Keränen, S. 1991. Cloning and sequencing of the yeast *Saccharomyces cerevisiae* *SEC1* gene localized on chromosome IV. *Yeast* 7, 643–650.
- 15 Ammerer, G. 1983. Expression of genes in yeast using the *ADC1* promoter. *Methods Enzymol.* 101, 192–201.
- Baldari, C., Murray, J.A.H., Ghiara, P., Cesareni, G. and Galeotti, C.L. 1987. A novel peptide leader which allows efficient secretion of a fragment of human interleukin 1 β in *Saccharomyces cerevisiae*. *EMBO J.*, 6, 229–234.
- 20 Becherer, K.A. and Jones, E.W., 1992. Role of the *PEP12* gene product in vacuolar targeting in yeast. *EMBL Data Bank* 31, accession number M90395.
- 25 Bennett, M.K., Calakos, N. and Scheller, R.H. 1992. Syntaxin: A synaptic protein implicated in docking of synaptic vesicles at pre synaptic active zones. *Science* 257, 255–259.
- 30 Buchert, J., Penttilä, M., Siika-aho, M., Saloheimo, A., Ranua, M. and Viikari, L. 1992. Mannanaasientsyymit, niitä koodittavat geenit ja menetelmä näiden eristämiseksi sekä menetelmä lignoselluloosapitoisen massan valkaisemiseksi (Mannanase enzymes, the encoding genes and method for their isolation, and a method for bleaching lignocellulose containing materials). *FI Pat. Appl.* 92 2373.
- 35 Buckholz, R.G. and Gleeson, M.A. 1991. Yeast systems for the commercial production of heterologous proteins. *Bio/Technology* 9, 1067–1072.
- 40 Dunn-Coleman, N., Bloebaum, P., Berka, R., Bodie, E., Robinson, N., Armstrong, G., Ward, M., Przetak, M., Carter, G., LaCost, R., Wilson, L., Kodama, K., Baliu, E., Bower, B., Lamsa, M. and Heinsohn, H. 1991. Commercial levels of chymosin production by *Aspergillus*. *Bio/Technology* 9: 976–981.
- 45 Fawell, E., Hook, S. and Armstrong, J., 1989. Nucleotide sequence of a gene encoding a YPT1-related protein from *Schizosaccharomyces pombe*. *Nucl. Acid Res.* 11, 4373.

- Frischauf, A.-M., Lerach, H., Poutstka, A. and Murray, N., 1983. Lambda replacement vectors carrying polylinker sequences. *J. Mol. Biol.* 170, 827-842.
- 5 Gerst, F.E., Rodgers, L., Riggs, M. and Wigler, M. 1992. *SNC1*, a yeast homolog of the synaptic vesicle-associated membrane protein/synaptobrevin gene family: Genetic interactions with the *RAS* and *CAP* genes. *Proc. Natl. Acad. Sci. USA* 89, 4338-4342.
- 10 Greenberg, G., Shelness, G.S. and Blobel, G., 1989. A subunit of mammalian signal peptidase is homologous to yeast *SEC11* protein. *J. Biol. Chem.* 264, 15762-15765.
- Hallborn, J., Penttilä, M., Ojamo, H., Keränen, S. & Hahn-Hägerdal, B. 1990. Xylose utilization by recombinant yeasts. *International Pat. Appl.* WO 91/15588.
- 15 Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166, 557-580.
- Hardwick, K.G. and Pelham, H.R.B. 1992. *SED5* encodes a 39 KD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *EMBL Data Bank* 32, accession number X66980.
- 20 Harkki, A., Uusitalo, J., Bailey, M., Penttilä, M. & Knowles, J.K.C. 1989. A novel fungal expression system: secretion of active calf chymosin from the filamentous fungus *Trichoderma reesei*. *Bio/Technology* 7: 596-603.
- 25 Haubruck, H., Prange, R., Vorgias, C. and Gallwitz, D. 1989. The ras-related mouse *ypt1* protein can functionally replace the YTP1 gene product in yeast. *EMBO J.* 8, 1427-1432.
- 30 Hirai, Y., Takebe, K., Takashina, M., Kobayashi, S. and Takeichi, M. 1992. Epimorphin: a mesenchymal protein essential for epithelial morphogenesis. *Cell* 69, 471-481.
- Inoue, A., Obata, K. and Agakawa, K. 1992. Cloning and sequence analysis of cDNA for a neuronal cell membrane antigen, HPC-1. *J. Biol. Chem.* 267, 10613-10619.
- 35 Irani, M.H. and Kilgore, T.L. 1988. High level expression in yeast. European patent application EP 0 284 044 A1.
- 40 Ito, H., Fukuda, Y., Murata, K. and Kimura, A. 1983. Transformation of intact yeast cells with alkali cations. *J. Bacteriol.* 153, 163-168.
- 45 Jeenes, D., MacKenzie, D., Roberts, I. and Archer, D. 1991. Heterologous protein production by filamentous fungi. *Biotechnology and Genetic Engineering Reviews*, vol. 9. Pp. 327-367.

- Keränen, S. 1986. Synthesis and processing of Semliki forest virus polypeptide in *Saccharomyces cerevisiae*: a yeast type glycosylation of E1 envelope protein. *Gene* 48, 267-275.
- 5 Lamsa, M. and Bloebaum, P. 1990. Mutation and screening to increase chymosin yield in a genetically-engineered strain of *Aspergillus avamori*. *J. Ind. Microbiol.* 5, 229-238.
- 10 Lopez, M.C., Nicand, J.M. and Gaillardin, C., 1992. SEC14 deleted mutant of *Yarrowia lipolytica* is altered in the secretion and differentiation processes. 16th International Conference on Yeast Genetics and Molecular Biology. Vienna, Austria, Aug. 15-21, 1992, *Yeast* 8 (Spec. Issue) p. 473.
- 15 Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. *Molecular Cloning, A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Martegani, E., Forlani, N., Mauri, I., Porro, D., Schleuning, W.D. and Alberghina, L. 1992. Expression of high levels of human tissue plasminogen activator in yeast under the control of an inducible GAL promoter. *Appl. Microbiol. Biotechnol.* 37, 604-608.
- 20 McKnight, G.L. and McConaughy, B.L.. 1983. Selection of functional cDNAs by complementation in yeast. *Proc. Natl. Acad. Sci. USA* 80, 4412-4416.
- 25 Mäntylä, A., Rossi, H., Vanhanen, S., Penttilä, M., Suominen, P. and Nevalainen, H. 1992. Electrophoretic karyotyping of wild type and mutant *Trichoderma reesei* strains. *Current Genetics* 21:471-477.
- 30 Novick, P. and Scheckman, R., 1979. Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 76, 1858-1862.
- Novick, P., Ferro, S. and Scheckman, R. 1981. Order of events in the yeast secretory pathway. *Cell* 25, 461-469.
- 35 Novick, P., Fields, C. and Scheckman, R. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21, 205-215.
- 40 Nyysönen, E., Keränen, S., Penttilä, M., Takkinen, K. and Knowles, J. K. C. 1990. Immunoglobulin production by *Trichoderma*. *US Pat. Appl.* 552757.
- Nyysönen, E., Penttilä, M., Harkki, A., Saloheimo, A., Knowles, J.K.C. and Keränen, S. 1993. Efficient production of antibodies by the filamentous fungus *Trichoderma reesei*. *Bio/Technology* 11, 591-595.
- 45

- Penttilä, M.E., Nevalainen, H., Rättö, M., Salminen, E. and Knowles, J.K.C. 1987. A versatile transformation system for the filamentous fungus *Trichoderma reesei*. *Gene* 61, 155-164.
- 5 Protopopov, V., Govindan, B., Novick, P. and Gerst, J.E. 1993. Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S. cerevisiae*. *Cell* 74, (in press).
- 10 Romanos, M.A., Scorer, C.A. and Clare, J.J. 1992. Foreign gene expression in yeast: a Review. *Yeast* 8, 423-488.
- Ruohonen, L., Hackman, P., Lehtovaara, P., Knowles, J.C.K. and Keränen, S. 1987. Efficient secretion of *Bacillus amyloliquefaciens* α -amylase by its own signal peptide in *Saccharomyces cerevisiae* host cells. *Gene* 59, 161-170.
- 15 Ruohonen, L., Penttilä, M. and Keränen, S. 1991. Optimization of *Bacillus* α -amylase production by *Saccharomyces cerevisiae*. *Yeast* 7, 337-346.
- 20 Sakai, A., Shimizu, Y. and Hishinuma, F. 1988. Isolation and characterization of mutants which show an oversecretion phenotype in *Saccharomyces cerevisiae*. *Genetics* 119, 499-506.
- Schuster, J.R., Moyer, D.L., Lee, H., Dennis, A., Smith, B. and Merryweather, J.P. 1989. *Gene* 83, 47-55.
- 25 Segev, N., Mulholland, J. and Botstein, D., 1988. The yeast GTP-binding YTP1-protein and a mammalian counterpart are associated with the secretion machinery. *Cell* 52, 915-924.
- 30 Sikorski, R.S. and Hieter, P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19-27.
- 35 Sleep, D., Belfield, G.P., Ballance, D.J., Steven, J., Jones, S. Evans, L.R., Moir, P.D. and Goodey, A.R. 1991. *Saccharomyces cerevisiae* strains that overexpress heterologous proteins. *Bio/Technology* 9, 183-187.
- Smith, R.A., Duncan, M.J. and Moir, D.T. 1985. Heterologous protein secretion from yeast. *Science* 229, 1219-1224.
- 40 Suzuki, K., Ichikawa, K. and Jigami, Y. 1989. Yeast mutants with enhanced ability to secrete human lysozyme: Isolation and identification of a protease-deficient mutant. *Mol. Gen. Genet.* 219, 58-64.
- 45 Vanoni, M., Porro, D., Martegani, E. and Alberghina, L. 1989. Secretion of *Escherichia coli* β -galactosidase in *Saccharomyces cerevisiae* using the signal

sequence from the glucoamylase-encoding *ST42* gene. Biochem. Biophys. Res. Commun. 164, 1331-1338.

- 5 Ward, M., Wilson, L.J., Kodama, K.H., Rey, M.W. and Berka, R.M. 1990. Improved production of calf chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion.

- 10 Wilson, D.W., Wilcox, C.A., Flynn, G.C., Chen, E., Unang, W-J., Henzel, W.J., Block, M.R., Ullrich, A. and Rothman, J.E., 1989. A fusion protein required for vehicle-mediated transport in both mammalian cells and yeasts. Nature 339, 355-359.

- 15 Zagursky, R.J., Berman, M.L., Baumeister, K. and Lomax, N. 1986. Rapid and easy sequencing of large linear double stranded DNA and supercoiled plasmid DNA. Gene Anal. Techn. 2, 89-94.

- 20 Zaraoui, A., Touchot, N., Chardin, P. and Tavitian, A. 1989. The human Rab genes encode family of GTP-binding proteins related to yeast YTP1 and SEC4 products involved in secretion. J. Biol. Chem. 264, 12394-12401.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Valtion teknillinen tutkimuskeskus
- (B) STREET: Vuorimiehentie 5
- (C) CITY: Espoo
- (E) COUNTRY: Finland
- (F) POSTAL CODE (ZIP): FIN-02150

(ii) TITLE OF INVENTION: Increased production of secreted proteins by recombinant eukaryotic cells

(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:

ATG AGT TAT AAT AAT CCG TAC CAG TTG GAA ACC CCT TTT GAA GAG TCA	48
Met Ser Tyr Asn Asn Pro Tyr Gln Leu Glu Thr Pro Phe Glu Glu Ser	
1 5 10 15	
TAC GAG TTG GAC GAA GGT TCG AGC GCT ATC GGT GCT GAA GGC CAC GAT	96
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
Lys Tyr Asp His Thr Ile Asn Gln Val Asp Ser Leu His Lys Arg Leu	
50 55 60	

30

CTG ACC GAA GTT AAT GAG GAG CAA GCA AGT CAC TTA AGG CAC TCC CTG	240
Leu Thr Glu Val Asn Glu Glu Gln Ala Ser His Leu Arg His Ser Leu	
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 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 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	

33

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

WE CLAIM:

1. An isolated DNA sequence of a *sec1* suppressor gene *SSO*, or a homologue thereof, which DNA sequence, when overexpressed in eukaryotic cells, renders the said cells capable to produce increased amounts of secreted proteins.
5
2. A DNA sequence according to claim 1, wherein the sequence is derived from a fungus, and when overexpressed in a fungal host, renders the said host capable to produce increased amounts of secreted proteins.
10
3. A DNA sequence according to claim 2 being selected from *SSO1* and *SSO2* sequences coding for a polypeptide comprising essentially the amino acid sequence given in SEQ ID NO: 2 and SEQ ID NO: 4, respectively, or a functional fragment thereof.
15
4. A vector comprising a DNA sequence according to any one of the claims 1 to 3.
20
5. A vector according to claim 4, which vector is capable of replicating autonomously when transformed into eukaryotic cells.
6. A vector according to claim 4, which vector is capable of integrating into the chromosome when transformed into eukaryotic cells.
- 25 7. A vector according to claim 4 being a yeast expression vector wherein the said DNA sequence is expressed under yeast gene regulatory regions.
8. A vector according to claim 7, wherein the said yeast gene regulatory regions are selected from the group consisting of the promoter regions of the *SSO1* gene, *SSO2* gene, *SEC1* gene, *GAL1* - *GAL10* genes, the alcohol dehydrogenase gene
30 *ADH1*, asparagine synthetase gene, and functional parts thereof.

9. A vector according to claim 4 being a filamentous fungus expression vector wherein the said DNA sequence is expressed under regulatory regions functional in filamentous fungi.
- 5 10. A vector according to claim 9, wherein the said regulatory regions functional in filamentous fungi are selected from the group consisting of the promoter regions of the *sso*, *cbh1*, *cbh2*, *egl1*, *egl2*, *tef1*, *pgk*, *pki*, glucoamylase, α -amylase and alcohol dehydrogenase genes.
- 10 11. A vector according to claim 4 which is a fungal vector selected from the group consisting of YEpSSO1 and YEpSSO2, structures of which are given in Fig. 1.
12. Recombinant eukaryotic cells carrying a DNA sequence according to any one of claims 1 to 3 and expressing enhanced levels of Sso protein(s).
- 15 13. Recombinant eukaryotic cells according to claim 12 being fungal cells belonging to a species 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.
- 20 14. Recombinant eukaryotic cells according to claim 13 being fungal cells belonging to a species selected from *Saccharomyces* and *Trichoderma*.
15. Recombinant eukaryotic cells according to claim 14 being fungal cells of
- 25 *Saccharomyces cerevisiae* strain VTT-C-92072 which has the deposition accession number DSM 7253.
16. Recombinant eukaryotic cells according to claim 14 being fungal cells of
- 30 *Saccharomyces cerevisiae* strain VTT-C-92073 which has the deposition accession number DSM 7254.

17. A method for constructing new eukaryotic cells capable of expressing enhanced levels of Sso protein(s), which method comprises:

- (a) isolating DNA sequence(s) coding for Sso protein(s) from a suitable donor organism;
- 5 (b) constructing a vector carrying at least one of the said DNA sequences; and
- (c) transforming at least one of the vectors obtained to suitable host cells.

18. A method according to claim 17, wherein the host to be transformed is selected from the group consisting of *Saccharomyces* spp., *Trichoderma* spp., *Kluyveromyces* spp., *Schizosaccharomyces pombe*, *Pichia* spp., *Hansenula* spp., *Yarrowia* spp.,
10 *Aspergillus* spp. and *Neurospora* spp.

19. A method according to claim 18, wherein said host belongs to a species selected from *Saccharomyces* and *Trichoderma*.

15

20. A process for producing increased amounts of secreted foreign or endogenous protein(s), by overexpressing the SSO gene(s) which process comprises:

- (a) isolating DNA sequence(s) coding for the said protein(s) from suitable donor organism;
- 20 (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
25 with enhanced production of the said protein(s); and
- (d) cultivating said recombinant host cells under conditions permitting expression of said protein(s).

21. A process for producing increased amounts of secreted foreign or
30 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(s) interacting with *SSO* gene, e.g. *SEC1* 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).

22. 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.

23. A process for efficient biomass production on a raw material or efficient hydrolysis of a raw material, which 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 to 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

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

24. A process 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),
10 which 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
15 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
20 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
25 expression of said hydrolytic enzyme(s).

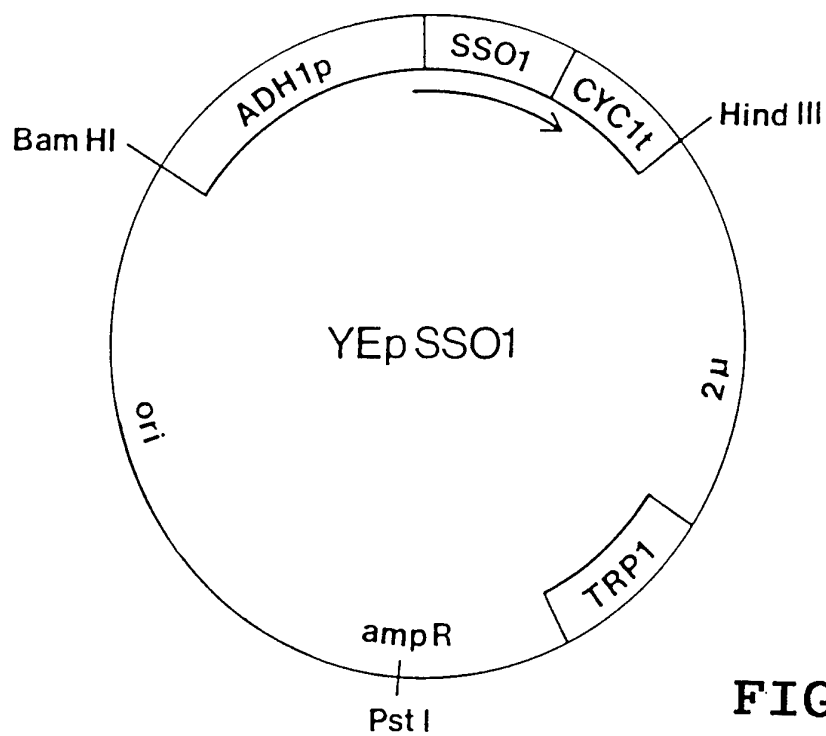


FIG. 1A

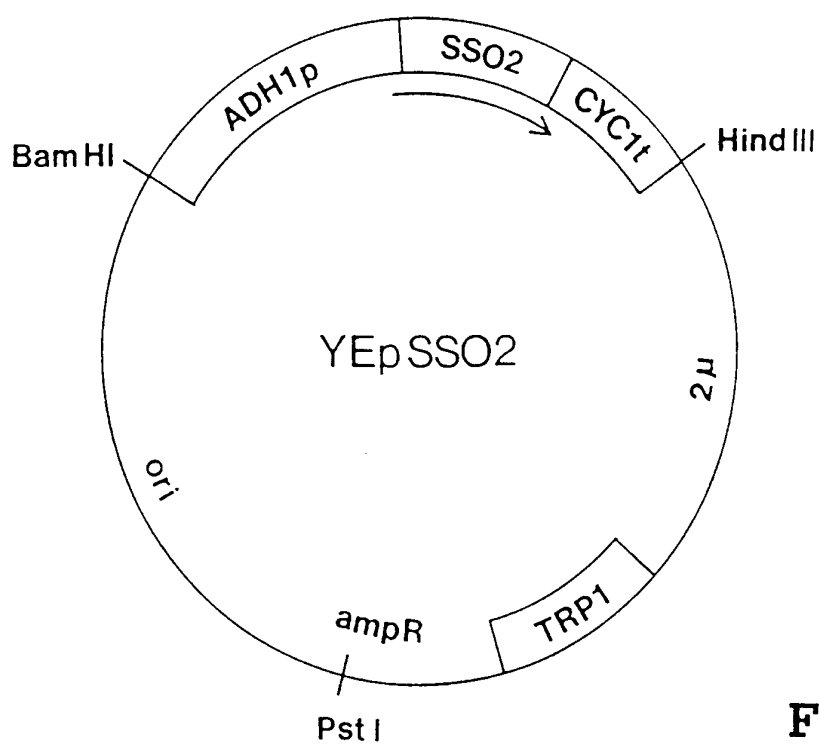


FIG. 1B

2/8

A B

Sso2p— 

FIG. 2

3/8

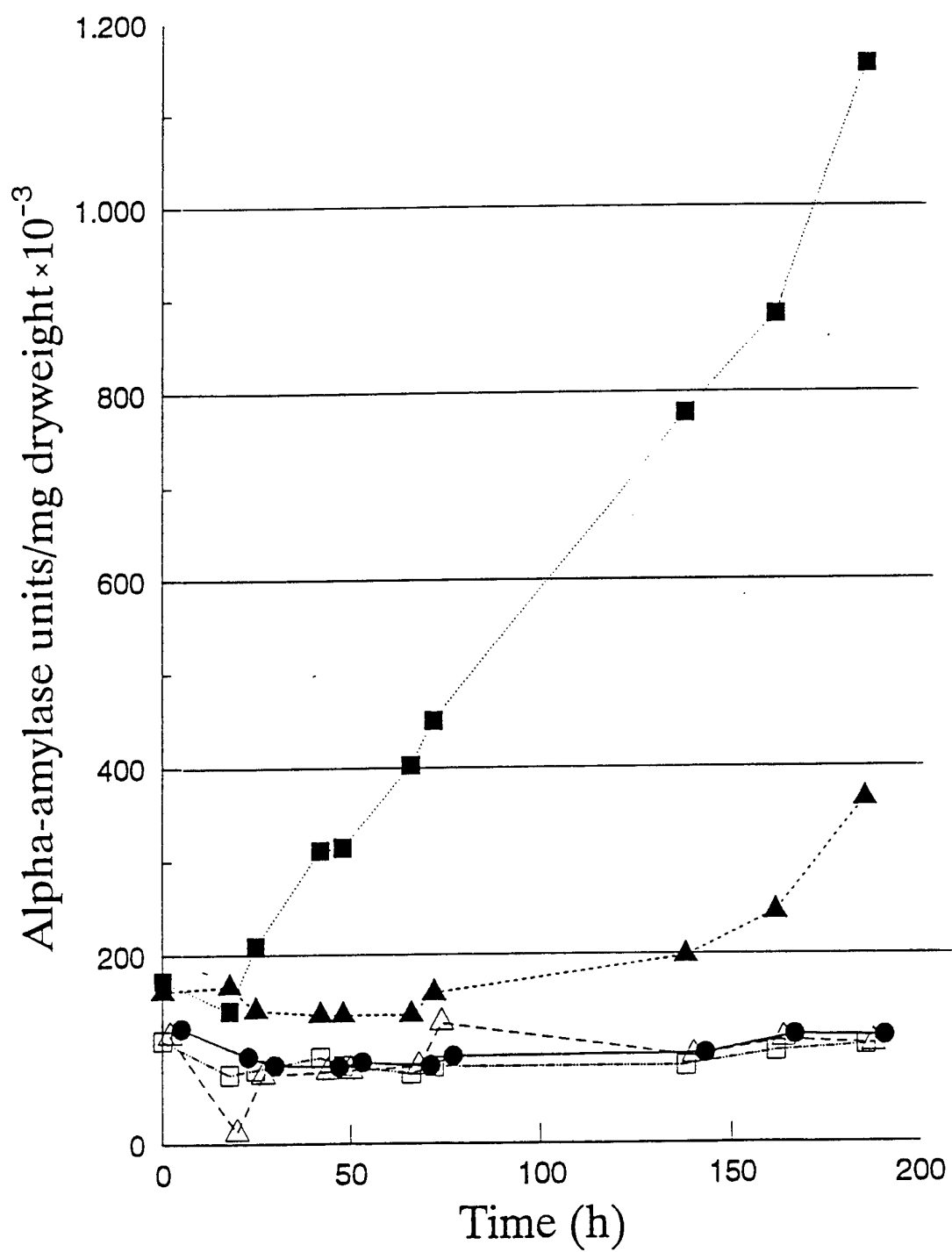


FIG. 3

4/8

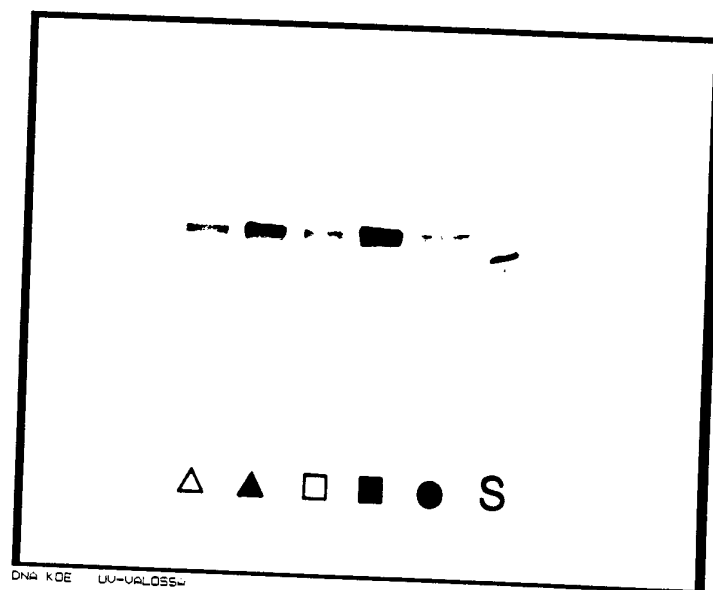


FIG. 4

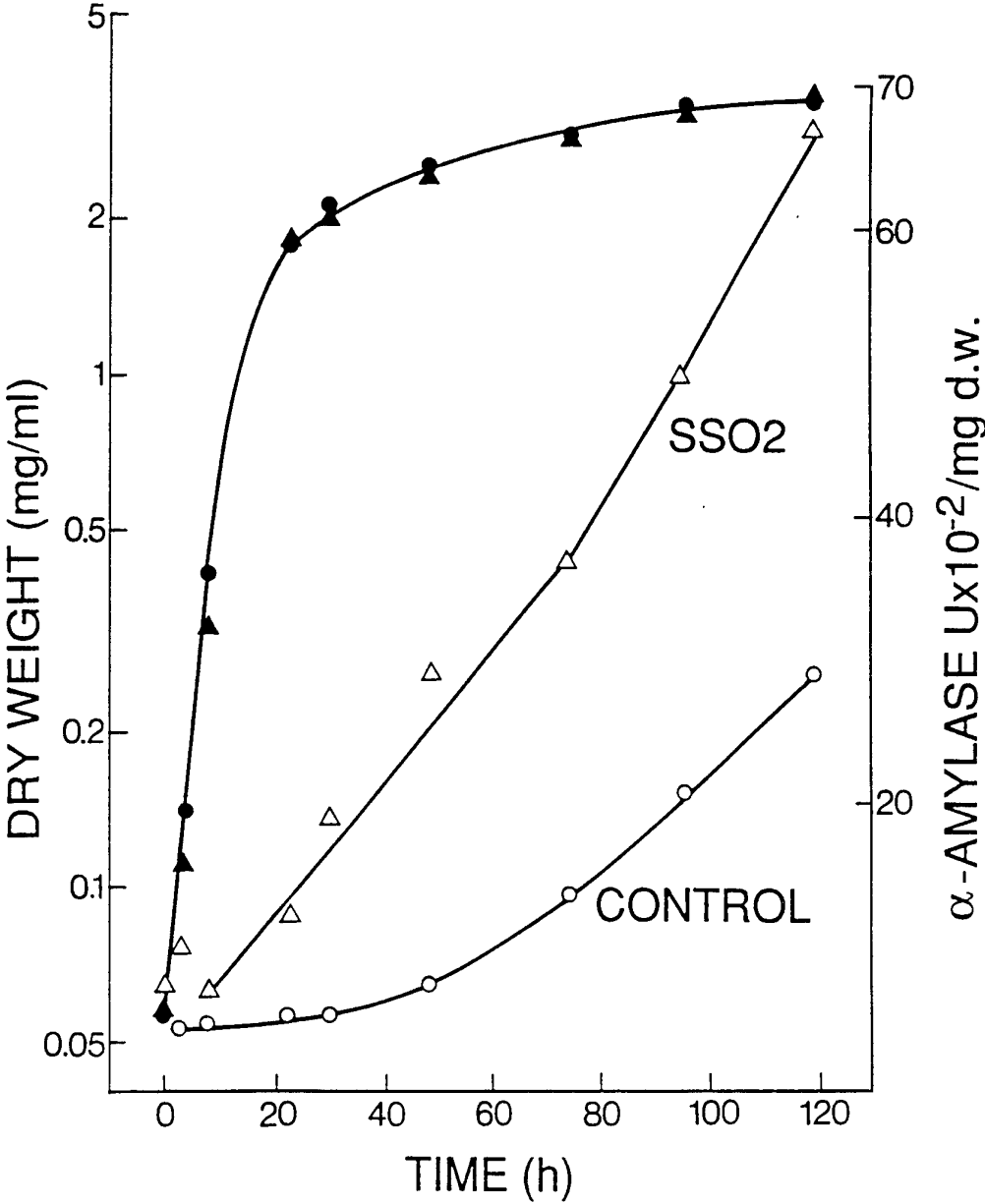
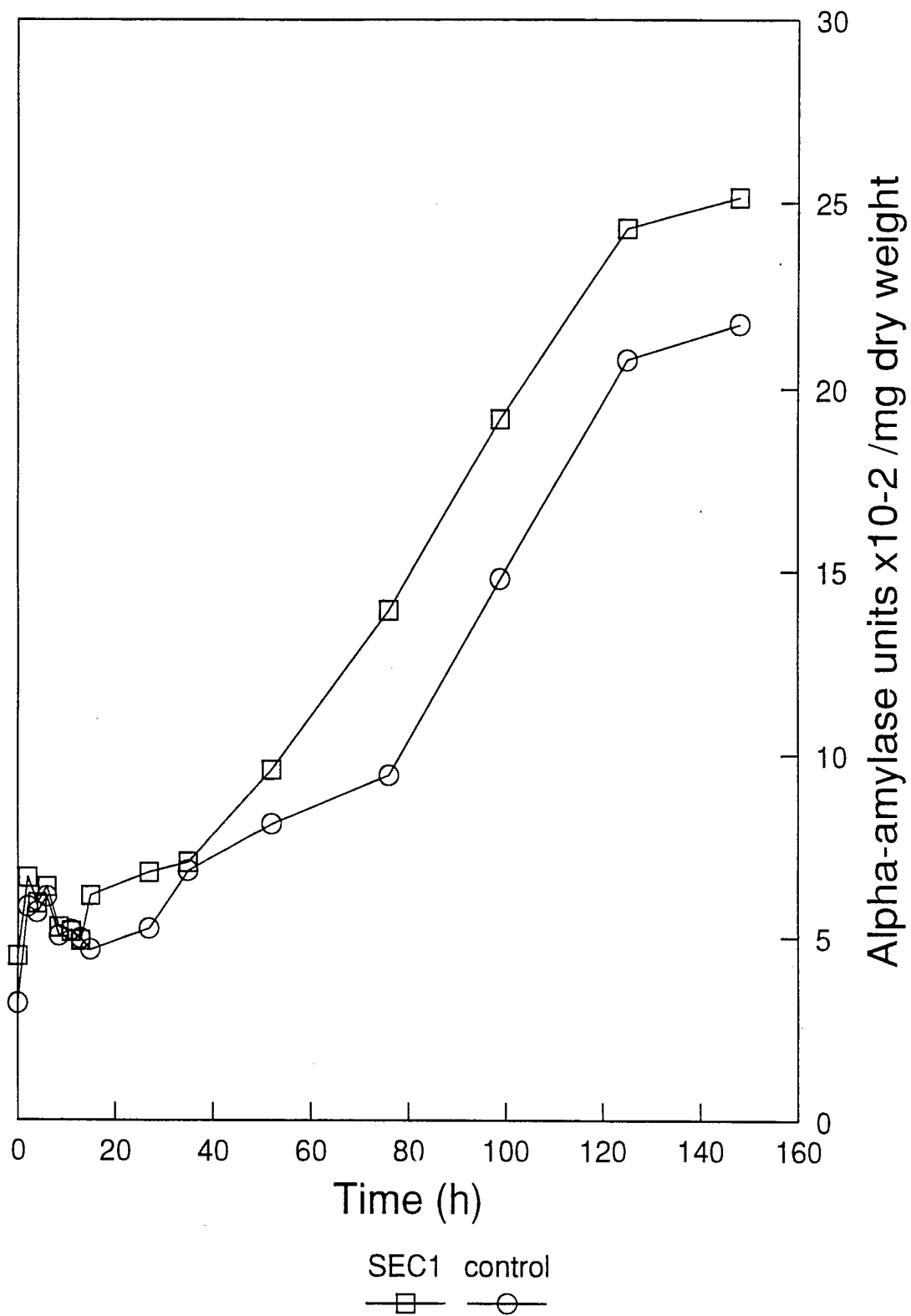
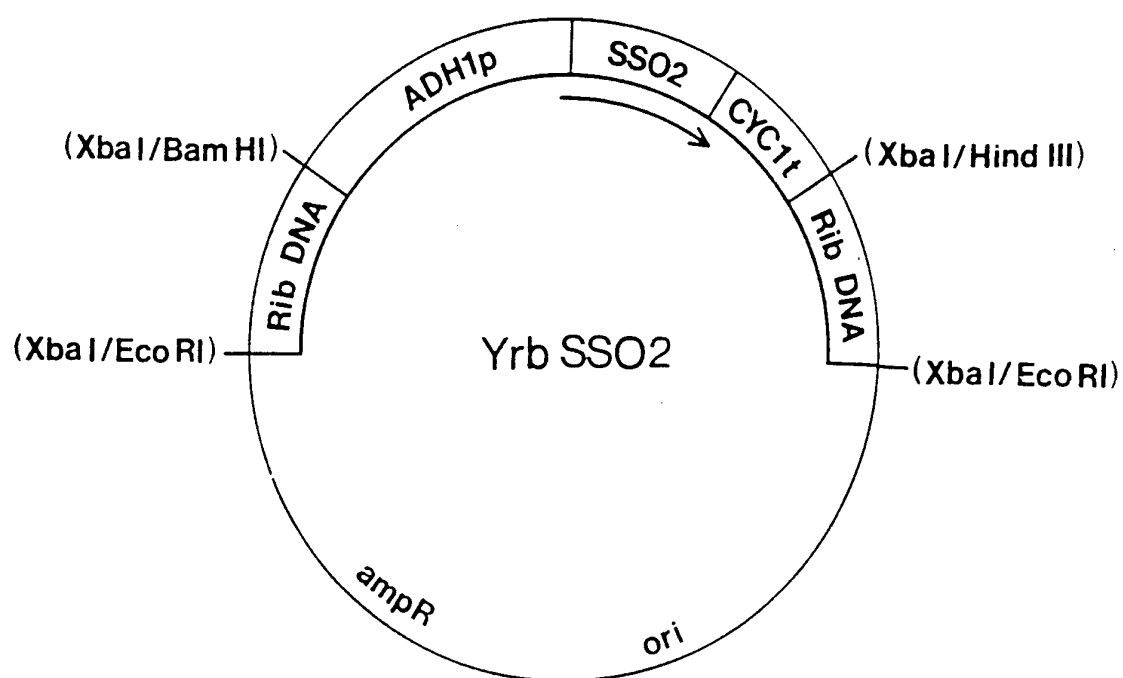


FIG. 5

6/8

**FIG. 6**

**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

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 93/00402

A. CLASSIFICATION OF SUBJECT MATTER		
IPC5: C12N 15/80, C12N 15/67, C12P 21/02 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC5: C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI, CA		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 267, No 15, May 1992, Akihiro Inoue et al, "Cloning and Sequence Analysis of cDNA for a Neuronal Cell Membrane Antigen, HPC-1", pages 10613 - pages 10619 --	1-24
A	Cell, Volume 69, May 1992, Yohei Hirai et al, "Epimorphin: A Mesenchymal Protein Essential for Epithelial Morphogenesis" page 471 - page 481 --	1-24
A	EP, A1, 0284044 (ZYMOGENETICS INC.), 28 Sept 1988 (28.09.88) --	1-24
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
18 January 1994		24-01-1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 93/00402

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Appl Microbiol Biotechnol, Volume 37, 1992, E. Martegani et al, "Expression of high levels of human tissue plasminogen activator in yeast under the control of an inducible GAL promoter" page 604 - page 608 -- -----	1-24

Information on patent family members

International application No.

PCT/FI 93/00402

Form PCT/ISA/210 (patent family annex) (July 1992)