A Process for the Production of Human Proinsulin in *Saccharomyces cerevisiae*

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Accepted for publication June 5, 1989

In order to develop a large-scale fermentation process for the production of human proinsulin in yeast, the intracellular expression of a human superoxide dismutase–human proinsulin fusion product (SOD–PI) has been studied. The expression of SOD–PI in *Saccharomyces cerevisiae* is regulated by a hybrid alcohol dehydrogenase 2/glyceraldehyde-3-phosphate dehydrogenase promoter. The promoter is repressed by glucose and derepressed by depletion of glucose. Although the genetic stability of the construction is shown to be poor under product-inducing conditions, it is demonstrated in shake flask experiments that a stable expression potential can be maintained in a complex medium for more than 60 generations by maintaining excess glucose throughout the cultivations. These results have been confirmed in continuous cultures in chemostat and turbidostat experiments. Addition of the glucose analogs glucosamine, 2-desoxyglucose, methylglucose, and thioglucose also leads to repression of SOD–PI formation. The analogs, however, are not suitable for improving genetic stability during propagation because of growth inhibition. In batch fermentation experiments in a complex medium at 30°C, it has been demonstrated that initial glucose concentrations up to 50 g/L result in high specific SOD–PI yields giving an overall yield of up to 700 mg SOD–PI/L whereas higher glucose concentrations lead to both lower specific and overall yields due to depletion of critical medium components in the production period. In fed-batch experiments at 30°C it has been possible to obtain high specific SOD–PI yields even at high biomass concentrations by feeding glucose at a constant rate of 1.5 g/L/h for 40 h followed by a feeding of ethanol at 1.0 g/L/h for 24 h, thus giving an overall yield of 1200 mg/L. Decreasing the temperature from 30 to 26°C leads to improved yields in batch as well as fed-batch experiments. The optimized fed-batch fermentation process which is suitable to be scaled up to the cubic meter level has been tested in 200-L fermentations resulting in yields of more than 1500 mg/L of the fusion protein which conveniently can be used as a precursor in the production of recombinant human proinsulin.

INTRODUCTION

Yeasts, especially *Saccharomyces cerevisiae*, have been used as expression systems for the production of heterologous proteins in a number of cases. This is mainly due to the extensive large-scale fermentation experience with this organism combined with the comprehensive knowledge of the genetic background and, furthermore, because of the absence of pyrogenic toxins. The prepro-α-factor leader region has in many cases proven to be a suitable signal for secretion of heterologous proteins in *S. cerevisiae*.[1–7] With the aim of producing insulin by in vitro conversion of proinsulin (PI) or proinsulin analogs, we (unpublished results) and others[5] have shown that proinsulin is only secreted in limited amounts using the α-factor system whereas some proinsulin analogs can be secreted in higher yields by removing or "hiding" the dibasic sites in the molecules which otherwise are cleaved by the yeast KEX 2 protease. Thus the α-factor system is a suitable system for the production of a precursor used in the production of insulin whereas it is less suitable in the production of proinsulin. The intracellular production of proinsulin in yeast has proven to be difficult because of proteolytic degradation (unpublished results). Furthermore the intracellular expression in yeast of a galactokinase–proinsulin fusion protein also resulted in low yields.[8]

The production of intracellular as well as periplasmic proinsulin or proinsulin fusion proteins in *Escherichia coli* have been extensively investigated.[9–15] In the periplasmic expression systems a rather poor yield is seen whereas a higher yield can be obtained in cytoplasmic expression systems. The main disadvantage of the *E. coli* systems is the difficulty of removal of endotoxins.

The scope of the work presented here is the development of a fermentation process resulting in high yields of proinsulin based on an intracellular expression system in *S. cerevisiae*. It has previously been shown that human superoxide dismutase (SOD) can be expressed intracellularly in yeast at very high levels.[16] This led to the idea that a SOD–PI fusion protein might also be expressed in high yields.[17] The two proteins were linked by a methionine group which could later be cleaved by cyanogenbromide, since proinsulin contains no methionine residues. The expression of the gene was controlled by an ADH2–GAPDH hybrid promoter, which is repressed by glucose and derepressed by depletion of glucose.[17,18]

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Biotechnology and Bioengineering, Vol. 35, Pp. 339–348 (1990)

CCC 0006-3592/90/040339-10$04.00

In this article the genetic stability of this construction and the expression of SOD–PI is studied in batch and continuous culture systems under product-inducing and -non-inducing conditions. These studies have led to the design of a propagation and of a fed-batch fermentation process which gives high expression levels of the fusion protein. The process has been scaled up to 200 L, giving a SOD–PI yield in excess of 1500 mg/L of culture.

MATERIALS AND METHODS

Media

YEP is a rich complex medium (10 g/L yeast extract, 20 g/L Bactopeptone, Difco, pH 5.0). In fermentation experiments 2 × YEP (i.e., twice as much of the ingredients as in YEP) is used supplemented with 2.5 g/L $MgCl_2$, 1.0 g/L $CaCl_2$, 1.0 g/L KCl, and 10 mL/L of a solution of trace metals (Zn, Fe, Mn, Cu, Co, B, Mo). In batch and shake flask experiments glucose was added at 30 g/L during the growth phase, and 20 g/L ethanol were added in the beginning of the production period. During fed-batch experiments glucose was added at a constant feeding rate at 1.5 g/L/h during propagation and ethanol was added at constant feeding rate at 1.0 g/L/h in the production period.

IS1 is a synthetic and selective medium without leucine, adjusted to pH 5.0, and composed of 10 g/L $(NH_4)_2SO_4$, 8.75 g/L KH_2PO_4, 1.25 g/L K_2HPO_4, 5.0 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L NaCl, 0.5 g/L $CaCl_2$, $2H_2O$, 10 mL/L 2*M* citric acid, trace metals, 40 mg/L of tryptophane, methionine, and arginine, 60 mg/L uracil, tyrosine, and lysine, 80 mg/L adenine, 100 mg/L phenylalanine, 160 mg/L histidine, and addition of growth factors (biotin, thiamine, pyridoxine, *meso*-inositol and calcium panthothenate). IS2 is IS1 supplemented with additional amino acids (50 mg/L of alanine, asparagine, aspartic acid, cystein, glutamine, glutamic acid, glycine, isoleucine, proline, serine, threonine, and valine) and growth factors (niacinamide, folic acid, riboflavin, and choline). IS1 and IS2 are only used for shake flask experiments, while YEP is used in both shake flasks and as a fermentation medium.

Yeast Strain and Culture Growth Conditions

The proinsulin gene was fused to the 3′ end of the gene encoding human superoxide dismutase (SOD) with methionine as linker between the two proteins.[17] The expression was controlled by using an ADH2–GAPDH hybrid promoter which was repressed by glucose and derepressed (i.e., product formation induced) in the absence of glucose.[17,18] The yeast plasmid pYASI1 contains this regulated expression system (ADH2–GAPDH–SOD–PI) as well as the *leu2* gene (introduced for selective purposes). From *Saccharomyces cerevisiae* strain 2150-2-3 (MATa, leu2-04, adel, cir°) an Ade+ revertant, PO17, was obtained.[17] This revertant was transformed with pYASI1 to leucine prototrophy and was maintained in glycerol at −80°C.

The glycerol stock culture was transferred to 100 mL medium in a 250-mL shake flask and incubated overnight at 30°C. Before further use it was checked that the cells were in exponential growth (i.e., glucose was in excess). This culture was used as inoculum for fermentation or shake flask experiments, the amount depending on the medium type: shake flasks containing YEP + 3% glucose were inoculated with 1 mL of a 1000-fold dilution, synthetic media were inoculated with 0.1–0.5 mL, and inducing medium was inoculated with 1 mL.

Subcultivations in shake flasks (for testing plasmid stability) were designed to keep the cells in exponential growth phase; i.e., the inoculation volumes and schedules were varied to secure a permanent glucose excess in order to avoid derepression of the ADH2–GAPDH promoter. Shake flasks were incubated at 30°C for 20–48 h.

Batch as well as fed-batch fermentations were inoculated with 2% inoculum (OD = 1–3). Batch fermentation experiments were run by adding 1–7% glucose before inoculation and 0–2% ethanol when production was initiated (see Results and Discussion). Fed-batch fermentations were fed with glucose at 1.5 g/L/h during propagation and with ethanol at 1.0 g/L/h during production.

Fermentation Equipment

Batch and fed-batch fermentation experiments were conducted in 2.5-L MBR-Mini-Bioreactors and in Chemap fermentors (7, 25, and 300 L) equipped with temperature, pH, air flow rate, and agitation controllers. All experiments were performed at pH 5.0 controlled by addition of 4.5*M* NaOH and 1.5*M* H_2SO_4. Air flow rate was constantly kept at 1 vvm, and agitation was regulated to ensure that the pO_2 level was above 40% of air saturation.

Chemostat and turbidostat experiments were conducted in a 2.5-L Chemap fermentor (working volume 1.0–1.5 L) with the equipment mentioned above as well as weight control and Fundalux (turbidity measurement and control on-line).

Analytical Methods

Glucose concentrations were semiquantitatively determined with sticks (Diabur-Test 5000, Boehringer-Mannheim) or quantitatively by Sigma kit no. 115 according to the recommended procedure. Ethanol concentrations in fermentation broth were determined by headspace gas chromatography. Fermentation broth turbidity was measured spectrophotometrically (optical density, OD, measured at 525 nm, in 1 cm cuvettes on a Spectronic 2000). OD_{525} = 1 corresponds to 0.25 g cell dry weight/L.

The yield of SOD–PI was estimated by extraction of harvested and washed cells and detected by C-peptide ELISA, SDS–PAGE, or FPLC analysis. The pellet of cells was resuspended in water, and after addition of glass beads (0.4–0.5 mm in diameter) the cells were disrupted in a shaker at 2200 rpm for 60 min at 4°C. The resulting ho-

mogenate contained cell debris and insoluble SOD–PI. The extraction and solubilization method of the fusion protein depended on the analytical detection method. For ELISA analysis the homogenate was treated with 6*M* guanidinium hydrochloride whereafter tris buffer at pH 8.0 was added. For SDS–PAGE analysis the homogenate was boiled for 5 min with equal volumes of sample buffer (containing 0.135*M* tris-HCl, pH 6.8, 6% SDS, 20% glycerol, 0.01% bromophenol blue, 100 m*M* DTT). For FPLC analysis 8*M* urea containing 10 m*M* DTT, pH 2.6, was added to the homogenate, and after centrifugation the supernatant was applied on a MonoQ HR 5/5 column from Pharmacia. SOD–PI was eluted by a NaCl gradient (0–400 m*M*) in 10 m*M* histidine hydrochloride, 7*M* urea, pH 6.0.

The fraction of plasmid-containing cells was determined as colony counts on selective plates (Yeast Nitrogen Base from Difco with addition of glucose and without leucine) and related to total colony counts on nonselective plates (YEP + glucose and with leucine).

Measurement of expression potential was carried out in shake flasks containing YEP + 2% ethanol which were inoculated with noninduced, exponentially growing culture and incubated at 30°C for 24 h before the yield of SOD–PI was measured by ELISA analysis.

RESULTS AND DISCUSSION

In designing a large-scale fermentation process using a recombinant microorganism expressing a heterologous protein, it is essential to consider the maintenance of plasmids during the process and to investigate the regulation of product formation. To maintain the plasmid content in the yeast cells, the leucine selection principle was adopted. As the host strain itself is leucine auxotroph, the *LEU2* gene was placed on the plasmid to make the transformed strain leucine prototroph, which means that only cells containing the *LEU2* gene will grow on a medium lacking leucine. The design of the expression system producing SOD–PI is based on regulation of the ADH2–GAPDH promoter by glucose. This means that presence of glucose in the medium will repress the promoter, whereas absence of glucose will derepress the promoter, leading to induction of product formation. This feature makes it possible to design the fermentation process to ensure that product formation will occur only after propagation to a suitable cell density.

Genetic Stability

In the scaling up of a fermentation process to the cubic meter level, it is very important to ensure that the expression potential is maintained for at least about 40 generations (see Fig. 1). In experiments investigating the genetic stability in shake flasks, we maintained exponential growth during 40–70 generations by several subcultivations. The inoculum size of each step was designed to ensure that the cells were still growing exponentially after 20 h of incubation at 30°C.

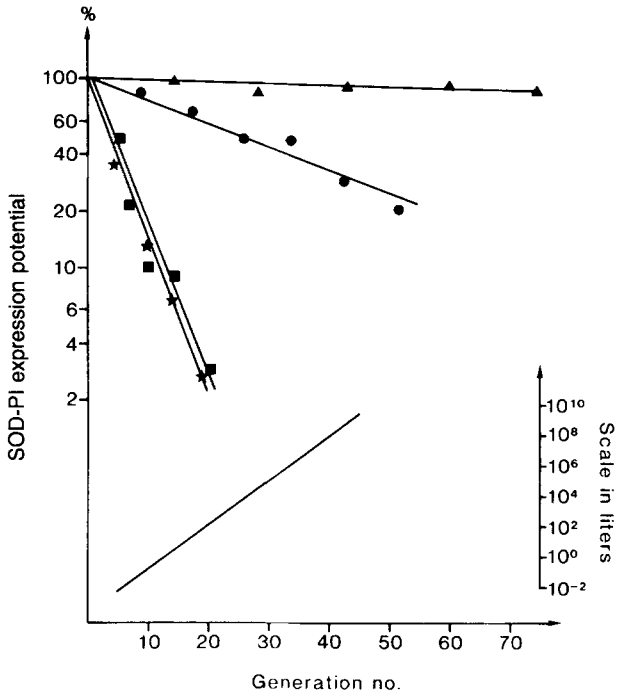

Figure 1. Genetic stability measured as conservation of product expression potential during subcultivations in shake flasks. The frozen master cell culture were defined as 0 generations and the specific SOD–PI expression of this was defined as 100% expression potential. The stability was investigated in different media: (*) IS1 (synthetic selective medium) + 2% ethanol; (■) YEP (rich nonselective medium) + 2% ethanol; (●) IS1 + 3% glucose; (▲) YEP + 3% glucose. Subcultivations in media with glucose were designed to keep glucose in excess throughout the experiments whereas the SOD–PI expression potential was measured by separate inoculations in media with ethanol and without glucose. At the bottom of the figure is shown how many generations will be required upon scale-up.

We measured the maintenance of SOD–PI expression during 20–70 generations in noninducing as well as inducing media. The expression potential was measured by removing a sample from the tested shake flask, inoculating another shake flask containing inducing medium (YEP + 2% ethanol), and incubating overnight at 30°C. It follows from this that the actual expression measured was the expression level after a few extra generations. However, as the procedure was always the same, the measured level was assigned to be the expression level at the time when the sample was taken. As a consequence the expression potential of a sample was measured relative to the expression potential of the master cell culture.

As shown in Figure 1, the expression potential decreased rapidly in inducing media (50% of the expression potential is lost after 3 generations and 90% after 10 generations). The type of medium (YEP or IS1) is not important as the stability in both types is rather poor. In contrast to these results, the composition of the noninducing media has great influence on the stability. Surprisingly, subcultivations in the rich, nonselective YEP medium result in better stability than in the selective, poorer medium. After about 30 generations the expression potential had decreased to 50% in

the selective medium, whereas good stability was seen in the YEP medium (after 60 generations, more than 90% of the expression potential was conserved). This led us to speculate whether growth in a rich medium with a high growth rate could improve the plasmid stability. In accordance with this hypothesis, it has previously been shown in both *E. coli* and in yeast that plasmid stability decreases when growth rate decreases.[19–21] Measurements of the specific growth rate in the different media revealed that the specific growth rate μ was 0.22 h^{-1} in IS1 or IS2 + 3% glucose and that μ was 0.55 h^{-1} in YEP + 3% glucose. When IS1 or IS2 is supplemented with leucine, the growth rate as well as the plasmid stability increased (data not shown), indicating that the plasmid stability is actually influenced by the growth rate rather than leucine selection.

It might seem surprising that although part of the cell population loses its expression potential (i.e., loss of plasmids), it is still able to grow in selective medium. This phenomenon could be explained either by the presence of leucine for a few generations after plasmid loss[22] or more likely due to reversion of the *leu2* mutation on the chromosome leading to the wild type genotype. Thus the leucine selection principle has only a minor effect on plasmid stability.

For further investigations of the plasmid stability during glucose limitation, a chemostat experiment with YEP + 1% glucose was designed in order to obtain steady-state conditions at $\mu = D = 0.22\ h^{-1}$ (Fig. 2). At this growth rate glucose is the limiting substrate component, and as a consequence, the hybrid promoter is derepressed and product formation is induced. In this case the product expression was measured directly in the samples and related to the expression potential of the master cell culture. However, the expression was rapidly decreasing, indicating a poor genetic stability in this system. The rate of decrease in expression was similar to the decrease of expression potential in the shake flask experiments with inducing media. This rate corresponds roughly to 20% loss per generation.

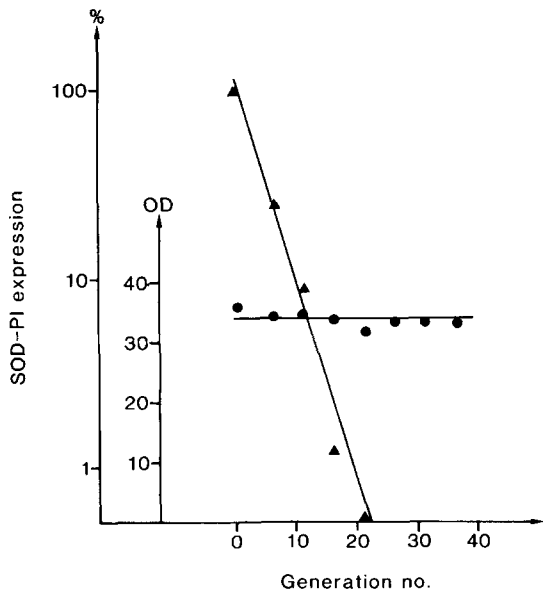

Figure 2. Glucose-limited chemostat experiment at $\mu = 0.22\ h^{-1}$. he concentration of glucose was 50 mg/L and the ethanol concentration was below 0.1% throughout the experiment. When steady-state conditions were reached, the population was defined as having a generation number of 0: (●) Biomass, OD; (▲) % SOD–PI expression.

It is demonstrated that initiation of product formation by derepression of the hybrid promoter leads to a rapidly decreasing expression potential. Hence, it is imperative to ensure effective repression of the hybrid promoter during propagation of the culture. One way to solve this problem could be to use glucose analogs as repressors during propagation. That these analogs, unlike glucose, cannot be metabolized by the yeast cells means that only a relatively small amount is needed during propagation. Shake flask experiments with the glucose analogs glucosamine, methylglucose, 2-desoxyglucose, and thioglucose were carried out in YEP + 2% ethanol to examine their repression efficiency during otherwise product-inducing conditions (Fig. 3). Only 2-desoxyglucose is effective in low concentrations (i.e, <0.1%; see Fig. 3, lanes 4 and 5); however, in addition, it appears to be growth inhibiting. The three other analogs are also repressing the promoter at higher concentrations, but these too become growth inhibiting (data not shown). Hence it was concluded that glucose analogs are not suitable for stabilizing the expression potential during propagation as the effective concentrations are both growth inhibiting and expensive. Alternatively, excess glucose during propagation must be considered. It is well known from chemostat experiments[23] that by increasing the dilution rate beyond a critical value, a shift from oxidative metabolism to fermentative metabolism is observed (the Crabtree effect). This shift is caused by an increase in glucose concentration with a medium-dependent critical dilution rate. In our system (YEP + 1% glucose) the shift was observed at dilution rates of about 0.3 h^{-1}. Therefore, to maintain glucose in excess in continuous cultures, it is necessary to obtain specific growth rates higher than this critical value.

It is possible to investigate fermentations at high growth rates (and high glucose levels) beyond the critical value at steady-state conditions in turbidostats. The advantage of the turbidostat compared to the chemostat in this context is the possibility to investigate substrate conditions (glucose in excess) at dilution rates close to the wash-out rate but without risking wash-out as would occur in chemostat experiments. In the turbidostat, the substrate addition (i.e., the dilution rate) is controlled by the turbidity of the culture. The turbidostat experiments were carried out at OD = 5 and at OD = 20 with YEP + 3% glucose as substrate (Fig. 4). The aim of these experiments was to study the genetic stability and the influence of glucose and ethanol concentration levels on the regulation of the hybrid promoter at specific growth rates higher than 0.3 h^{-1}, i.e., when glucose is not limiting. As shown in Figure 4A, the conditions at OD = 5 (resulting in $\mu = 0.52\ h^{-1}$) give rise to a high glucose concentration (2%) and a relatively low

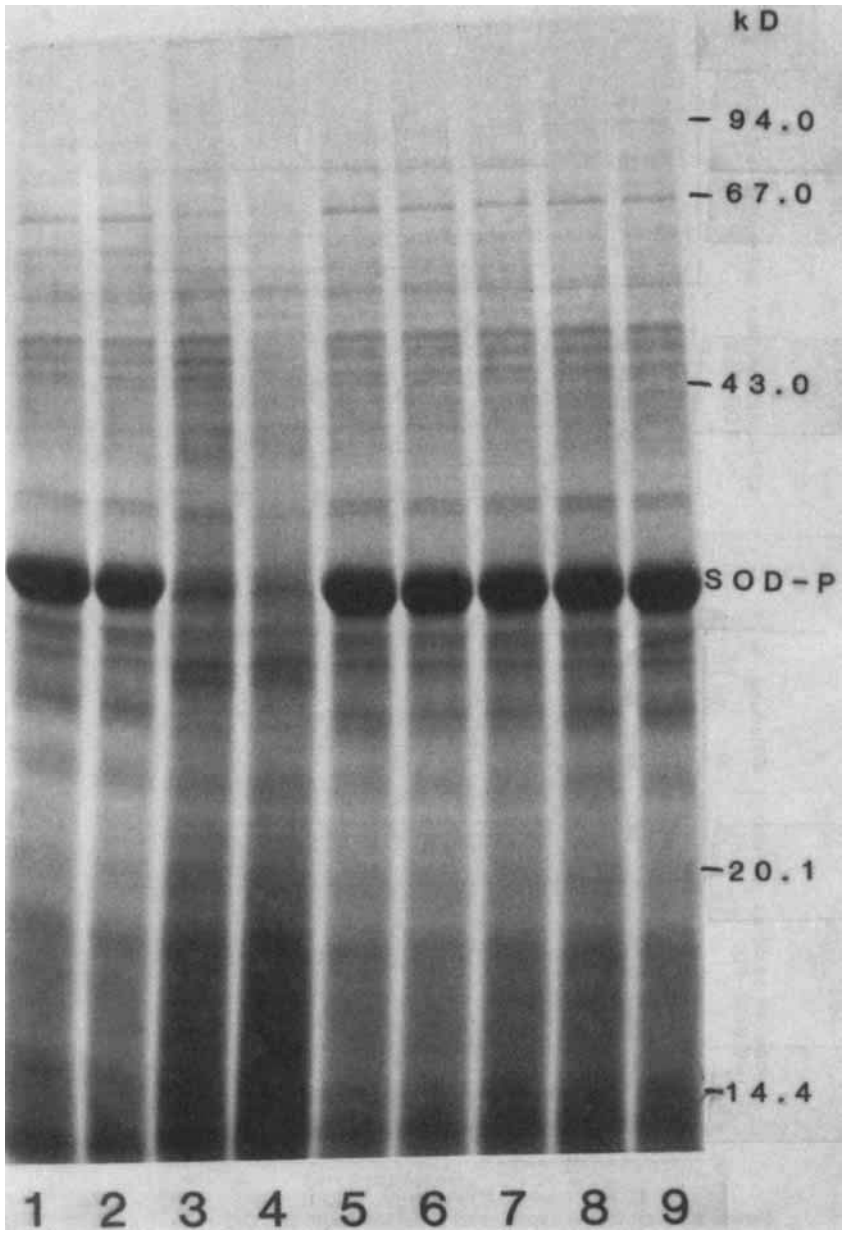

Figure 3. 12.5% SDS–PAGE of samples from shake flasks with product-inducing medium (YEP + 2% ethanol) containing glucose analogs in two different concentrations (0.05 or 0.1%). Each shake flask was inoculated with an exponentially growing culture. Lane 1, 0.05% glucosamine; lane 2, 0.1% glucosamine; lane 3, 0.05% 2-desoxyglucose; lane 4, 0.1% 2-desoxyglucose; lane 5, 0.05% methylglucose; lane 6, 0.1% methylglucose; lane 7, 0.05% thioglucose; lane 8, 0.1% thioglucose; lane 9, control without addition of glucose analogs.

ethanol concentration (0.4%). The product expression is strongly repressed, and consequently good genetic stability is observed throughout the experiment, i.e., for 70 generations. In the experiment at OD = 20 (resulting in $\mu = 0.35\ h^{-1}$) the glucose concentration is lower (1%) and the ethanol concentration higher (0.8%) than in the previous experiment. Even though these conditions give rise to a slightly higher product formation, the expression potential is maintained for at least 40 generations (see Fig. 4B). The conservation of expression potential was confirmed by a constant plasmid copy number throughout the experiment in both cases (data not shown). At the end of the experiment at OD = 20 the medium addition was stopped and the system was run as a batch fermentation. Both the glucose and the ethanol concentrations were observed to decrease due to oxidative metabolism with resulting induction of product formation caused by the low glucose level. In accordance with previously described experiments the ex-

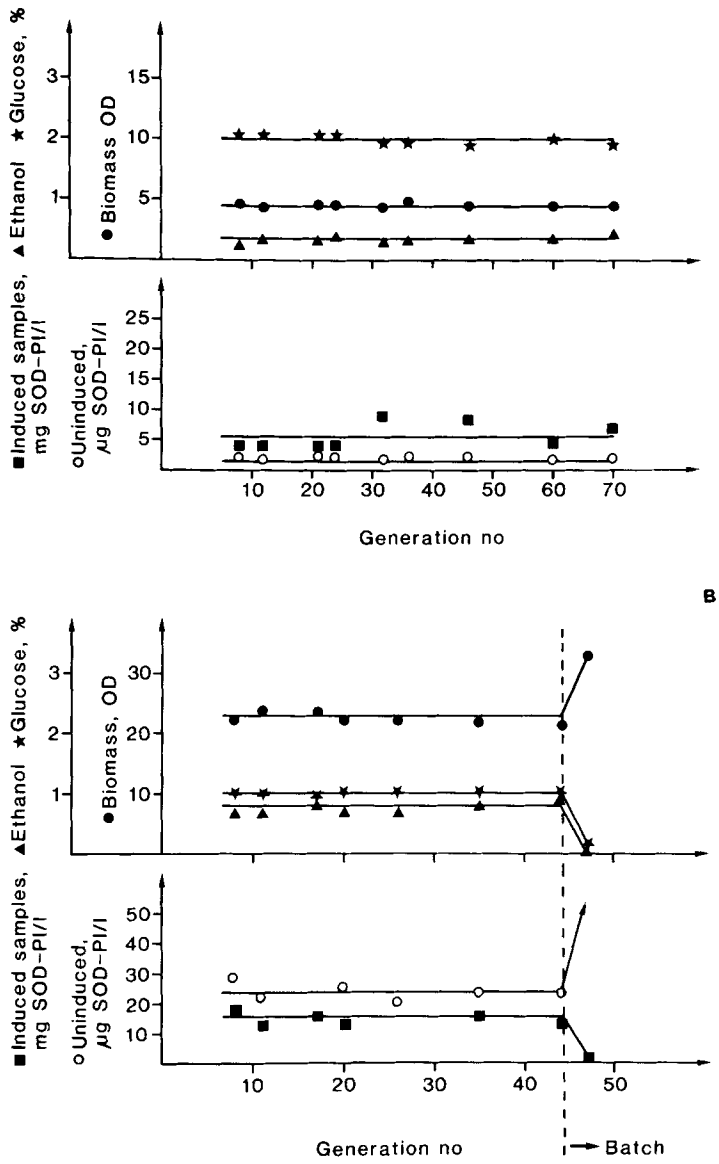

Figure 4. Turbidostat experiments at OD = 5 (A) and OD = 20 (B): (▲) % ethanol; (*) % glucose; (●) OD; (■) mg SOD–PI/L of induced samples; (○) µg SOD–PI/L of uninduced samples.

pression potential decreases severely after a few generations. It is thus demonstrated by subcultivations in shake flasks as well as by turbidostat experiments that the expression potential of the SOD–PI producing organism can be maintained for at least 60 generations in a rich medium containing excess glucose.

Fermentation Process Development

The first approach in the development of a fermentation process was to study simple batch fermentations with addition of increasing amounts of glucose (1–7%) before inoculation. The kinetics of biomass formation, glucose consumption, ethanol formation, specific SOD–PI accumulation, and total SOD–PI accumulation in batch fermentations with 5 and 7% glucose are shown in Figures 5A and 5B, respectively. During exponential growth glucose is consumed and ethanol formed as a result of the fermentative metabolism. When glucose is depleted from the medium, a secondary growth phase is observed after a lag phase due to a shift of carbon source metabolism from glucose to ethanol (diauxic growth). About 8–10 h after glucose depletion, product formation is initiated (Fig. 5A). At the end of the fermentation the specific yield is about 10 mg SOD–PI/L/OD and the overall yield is about 500 mg SOD–PI/L. [Later experiments (data not shown) yield up to 700 mg SOD–PI/L).] In contrast, when 7% glucose is added initially, very little SOD–PI formation is

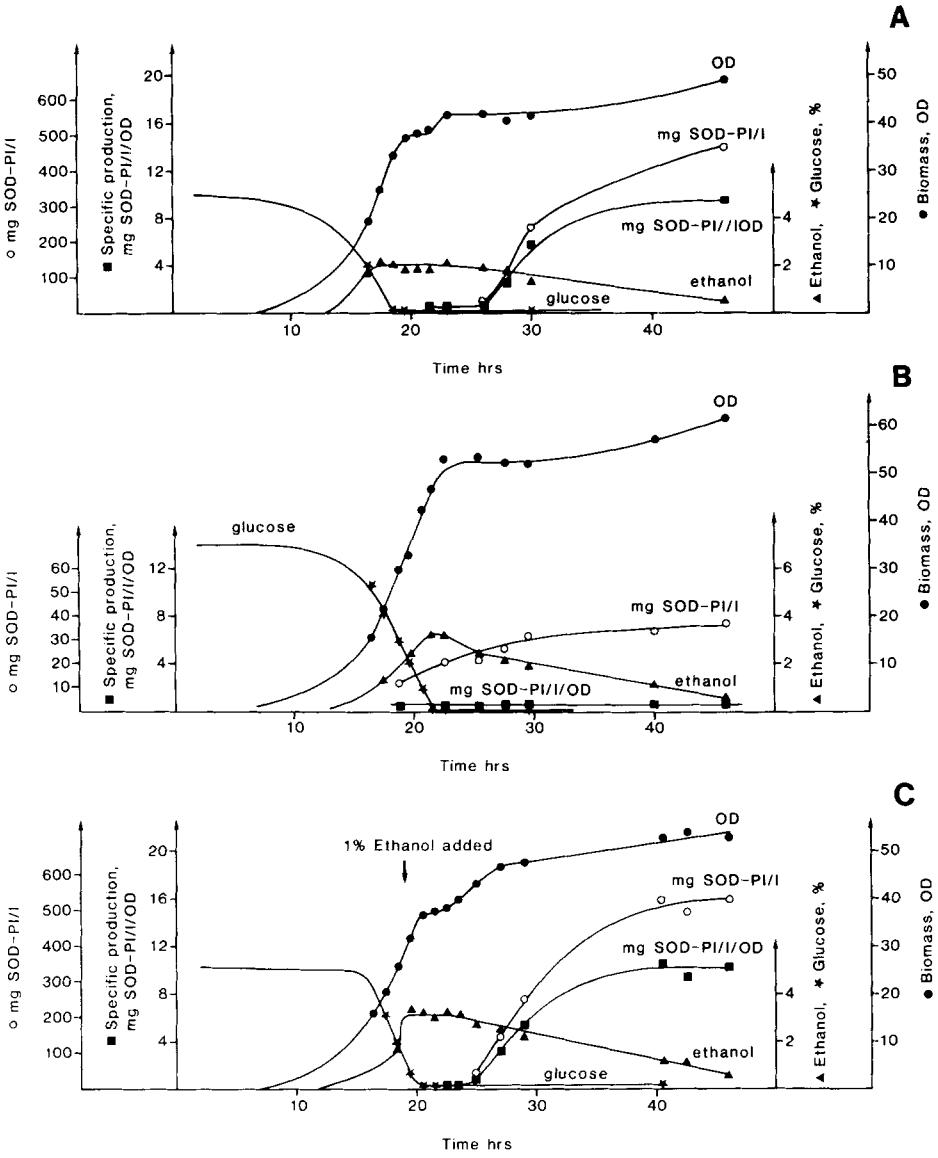

Figure 5. Kinetics of batch fermentation experiments: (●) OD; (■) mg SOD-PI/L/OD; (○) mg SOD-PI/L; (▲) ethanol concentration; (*) glucose concentration. (A) Batch fermentation with 5% glucose. (B) Batch fermentation with 7% glucose. (C) Identical to (A) but additionally 1% ethanol was added before glucose was depleted from the medium (after 19 h).

observed, although the expression potential measured during the fermentation did not decrease (data not shown). To investigate whether the lack of product formation was due to inhibiting concentrations of ethanol, a similar set of batch fermentation experiments with 1–7% glucose was set up with addition of 1% ethanol when the glucose level was decreasing rapidly. The results of a batch fermentation with and 1% ethanol addition are shown (Fig. 5C) to be almost identical to the results of the corresponding fermentation with 5% glucose but without ethanol addition. These data demonstrate that the ethanol concentration in Figure 5B is not inhibiting for SOD–PI expression. The reason for the significant decrease in product formation when the high glucose concentration is used might be that the extra biomass formation causes depletion of critical substrate components which are essential for the synthesis of the fusion protein. This hypothesis was confirmed experimentally using an even more concentrated YEP medium plus 7% glucose in which the expression potential could be at least partially restored (data not shown).

In Figure 6A, the yields of biomass, ethanol, and specific product formation as well as overall product formation are depicted as a function of the amount of glucose added. Figure 6B shows the similar results when 1% extra ethanol is added. In both cases the specific yields as well as total yields of SOD–PI decrease rapidly when more than 5%

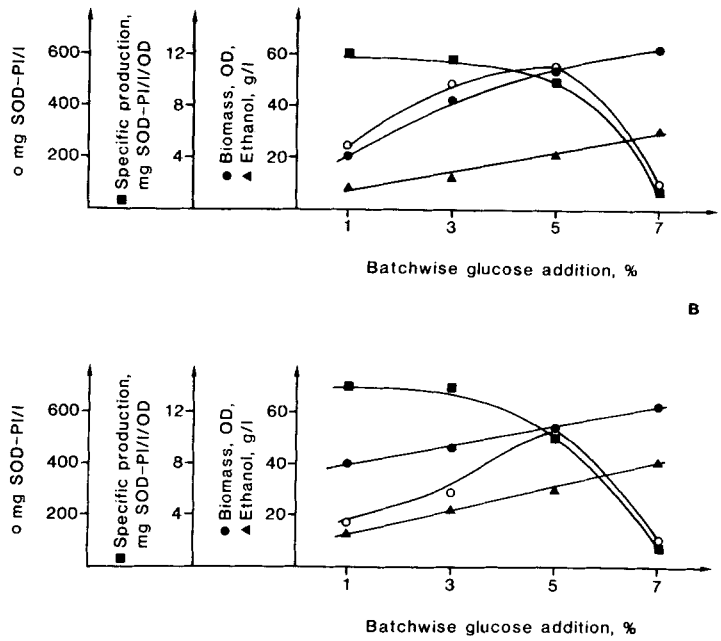

Figure 6. (A) Product formation in batch fermentations as function of amount of glucose added batchwise. (B) Product formation as a function of added amount of glucose in batch fermentations which have been supplemented with 1% ethanol before glucose was depleted from the medium. Symbols as in Figure 5.

glucose is added, indicating that in batch fermentations it is not possible simultaneously to obtain a high biomass formation and a high product formation.

During fermentative metabolism which is prevailing in batch fermentations the biomass yield coefficient for glucose, $Y_{x/s}$, is low, and ethanol is accumulated. This leads to inefficient utilization of the substrate and eventually to growth inhibition at very high glucose or alcohol concentrations. To obtain high biomass concentrations without these drawbacks, the metabolism of glucose should be changed from fermentative to oxidative as the yield coefficient, $Y_{x/s}$, for the oxidative metabolism is higher and only small amounts of ethanol are formed. However, to obtain oxidative metabolism, it is necessary to keep the specific growth rate below 0.3 h^{-1} (see discussion of the turbidostat experiments), and this is possible by controlling the feed rate of glucose in a fed-batch fermentation. It is, however, a delicate matter to secure that induction of product formation is not initiated too early, as the system, as previously shown (see the section on genetic stability), is very unstable as soon as induction is initiated. To prevent induction, it is important to avoid glucose depletion so a fed-batch process should be designed in a way that glucose is kept in excess during the propagation period. On the other hand, glucose should be limited when product formation is required. One solution to these demands is the use of a constant glucose feed rate during the fermentation which initially gives rise to excess glucose when the biomass concentration is low and which at a later stage, when the increase in biomass concentration leads to a higher glucose consumption rate, results in glucose limitation and product induction. If the feed rate is too low, induction will begin at a low biomass concentration, resulting in low total yields because of genetic instability. When the feed rate is too high, the induction will occur late in the fermentation, and conditions similar to a batch fermentation with high glucose addition will be obtained (i.e., no product formation due to substrate limitations). Experiments where the feed rate was varied revealed that feed rates between 1 and 2 g glucose/L/h were optimal; a feed rate of 1.5 g/L/h was selected for further experiments. This feed rate results in glucose concentrations in excess of 0.1% until OD is 30 followed by a partial repression of the ADH2–GAPDH promoter during the next 8–10 h until OD is 80–100. The SOD–PI accumulation increases linearly and is proportional to the increase in biomass from OD = 80 to OD = 130 (Fig. 7A). However, the total SOD–PI concentration is similar to that from a batch fermentation (i.e., 700 mg/L), which results in lower specific yields for the fed-batch fermentation (5 mg SOD–PI/L/OD) compared to the batch fermentation (13 mg SOD–PI/L/OD). This is due to the higher biomass concentrations obtained in the fed-batch experiments.

To improve the specific yield of the fed-batch fermentations, the feed of carbon source was modified when SOD–PI production was initiated. Glucose was, as before, fed at a constant rate of 1.5 g/L/h during propagation, but after 40–45 h glucose addition was replaced by ethanol addition at 1.0 g/L/h. This adjustment resulted in an improved

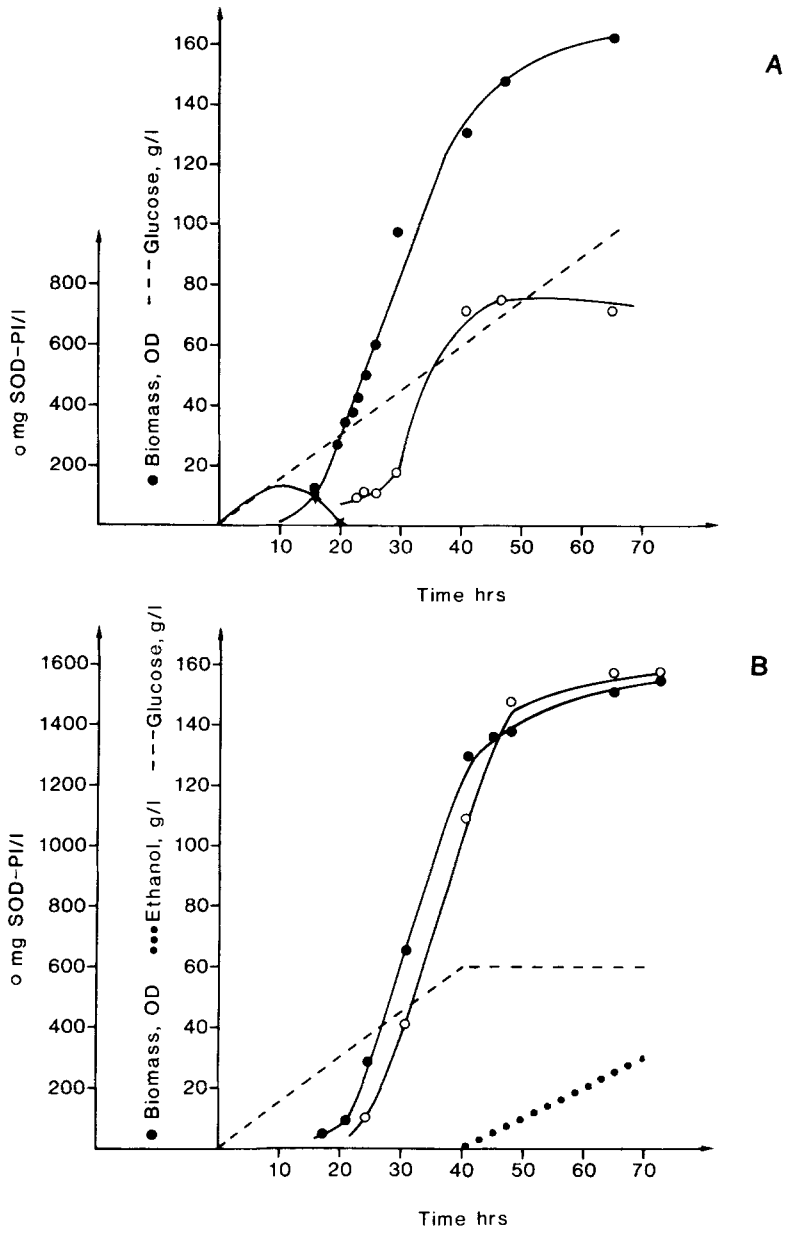

Figure 7. Kinetics of fed-batch fermentation experiments. (------) Accumulated addition of glucose. (· · · · · ·) Accumulated addition of ethanol. Other symbols as in Figure 5. (A) Fed-batch glucose 1.5 g/L/h, 30°C, pH 5.0. (B) Fed-batch glucose 1.5 g/L/h for 41 h and fed-batch ethanol 1.0 g/L/h for the next 31 h, 26°C, pH 5.0.

product concentration (1200 mg SOD–PI/L) and specific yields of 8 mg SOD–PI/L/OD. These results lead us to conclude that even though glucose is present only at low concentrations (less than 20 mg/L) late in the glucose fed-batch fermentation process, glucose or metabolite from the glycolysis is still able to partially repress the ADH2–GAPDH promoter. Furthermore, we have shown (Table I) that decreasing the fermentation temperature from 30 to 26°C results in even higher yields in both batch and fed-batch fermentations (i.e., up to 1600 mg SOD–PI/L). This might be due to decreased proteolytic activity at the lower temperature. In support for this hypothesis we have seen

Table I. Specific and absolute yields in batch and fed-batch fermentations at 26 and 30°C.

		26°C	30°C
Batch	OD	60	55
	mg SOD–PI/L	800	700
	mg SOD–PI/L/OD	13	13
Fed-batch glucose	OD	150	150
	mg SOD–PI/L	800	700
	mg SOD–PI/L/OD	5	5
Fed-batch glucose and ethanol	OD	150	150
	mg SOD–PI/L	1600	1200
	mg SOD–PI/L/OD	11	8

that incubation of a solution of SOD–PI with supernatant from homogenized cells at 26 and 35°C resulted in a more distinct degradation at the higher temperature (data not shown). Another reason for a better yield at the lower temperature might be the effect of temperature on the glucose repression/depression of the ADH2–GAPDH promoter.

The results presented in this report lead to preference of an optimized fed-batch fermentation process, although the genetic stability was initially considered to be better in a batch process. Detailed investigations of batch fermentations have revealed that it is not possible to maximize specific SOD–PI yields as well as high biomass concentrations simultaneously. As a consequence, a fed-batch process has been developed which resembles a batch process during the exponential propagation. The exponential growth phase is followed by a linear growth phase during which a partial repression of the ADH2–GAPDH promoter is seen, and finally, when a shift of carbon source from glucose to ethanol is performed, a complete derepression and a high product formation is obtained.

The optimized fed-batch fermentation at 26°C yielding about 1600 mg SOD–PI/L with a shift of carbon source after 41 h is shown in Figure 7B, corresponding to a specific yield of 11 mg/L/OD at an OD of 150. This process has been scaled up to a 200-L fermentation without any significant change in the product concentration.

Other fusion proteins involving the SOD molecule can be produced by the methods described in this report. As an example we have expressed fusions of SOD and various insulin analogs with results similar to those described here.

CONCLUSIONS

The intracellular expression of SOD–PI in *S. cerevisiae* can be maintained for more than 60 generations in a complex media by keeping glucose in excess, resulting in repression of the ADH2–GAPDH hybrid promoter.

A fed-batch fermentation process has been developed which ensures that formation of SOD–PI is repressed in the early fermentation period by feeding glucose at a constant rate for 40–45 h whereafter the product formation is initiated by replacing the glucose feed by an ethanol feed at a constant and relatively low rate. The yield can be further increased by lowering the fermentation temperature from 30 to 26°C. The optimized fermentation process leads to a yield of more than 1500 mg SOD–PI/L in a complex medium in a process which is suitable to be scaled up to the cubic meter level. The process has been run in 200-L fermentations with results similar to the bench-scale fermentations. Thus a large-scale fermentation procedure has been developed which results in a high yield of the fusion protein SOD–PI, giving a convenient raw material for the production of recombinant human proinsulin. The processes described in this report can also be used for the production of other fusion proteins involving SOD.

We thank our colleagues at Nordisk Gentofte A/S, especially Ib Groth Clausen, who made the analysis of plasmid copy no., Bente Bergquist for excellent technical assistance, and Britt Prieske for typing the manuscript. We also thank our colleagues at Chiron Corporation, who have contributed to this project.

NOMENCLATURE

ADH2	alcohol dehydrogenase II
C-peptide	connecting peptide between A- and B-chain in proinsulin
D	dilution rate (h^{-1})
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
OD	optical density at 525 nm
PI	proinsulin
SDS–PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
SOD–PI	fusion protein between superoxide dismutase and proinsulin
vvm	volume air/volume liquid/min
$Y_{x/s}$	biomass yield coefficient (g biomass/g substrate)
μ	specific growth rate (h^{-1})

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