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ORIGINAL PAPER

Plasmid stability and kinetics of continuous production of glucoamylase by recombinant *Saccharomyces cerevisiae* in an airlift bioreactor

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Abstract Production of glucoamylase by recombinant *Saccharomyces cerevisiae* C468/pGAC9 (ATCC 20690) in a continuous stirred tank bioreactor was studied at different dilution rates. Plasmid stability was found to be growth (dilution rate) dependent; it increased with the dilution rate. Bioreactor productivity and specific productivity also increased with the dilution rate. A kinetic equation was used to model the plasmid stability kinetics. The growth rate ratio between plasmid-carrying and plasmid-free cells decreased from 1.397 to 1.215, and segregational instability or probability of plasmid loss from each cell division decreased from 0.059 to 0.020 as the dilution rate increased from 0.10 to 0.37 1/h. The specific growth rates increased with dilution rate, while the growth rate difference between plasmid-carrying and plasmid-free cell populations was negligible. This was attributed to the low copy number of the hybrid plasmid pGAC9. Thus, the growth rate had no significant effect on plasmid instability. The proposed kinetics was consistent with experimental results, and the model simulated the experimental data well.

Keywords *Saccharomyces cerevisiae* · Glucoamylase · Kinetics · Dilution rate · Plasmid stability

List of symbols

D	Dilution rate (1/h)
F^+	Fraction of plasmid-carrying cells (%)
F_0^+	Initial value of F^+ at time $t = 0$ (%)
GA	Glucoamylase activity (U/l)
M	Ratio of N^- to N^+
M_0	Initial value of M at time $t = 0$
N	Cell density of total cell population (g/l)
N^+	Cell density of plasmid-carrying cells (g/l)
N^-	Cell density of plasmid-free cells (g/l)
P	Productivity (g/l/h)
R	Specific plasmid loss rate: $R = \mu^+\Phi$ (1/h) or (1/gen)
t	time (h)

Greek symbols

β	Probability of plasmid loss during each cell division; $\beta = R/\mu^+$
τ_0	Growth rate ratio; $\tau_0 = \mu^-/\mu^+$
μ^+	Specific growth rate of plasmid-carrying cells (1/h)
μ^+_{max}	Maximum Specific growth rate of plasmid-carrying cells (1/h)
μ^-	Specific growth rate of plasmid-free cells (1/h)
μ^-_{max}	Maximum Specific growth rate of plasmid-free cells (1/h)
μ_{app}	Apparent growth rate in suspension cells (1/h)
γ	specific glucoamylase formation rate (U/g cells/h)
σ^2	Variance

Introduction

The yeast *Saccharomyces cerevisiae* grows rapidly in a relatively simple medium to high densities with a high level of product secretion. It also demonstrates a eukaryotic subcellular organization capable of some posttranslational modifications, which are required for the integrity of most proteins. Moreover, this yeast produces not only a reasonable amount of recombinant proteins, but also secretes the proteins outside the cell envelope, which reduces the

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cost of downstream processing. Furthermore, the amount of secreted by-products such as proteases is low under non-selective conditions. *S. cerevisiae* is also widely used for commercial production of ethanol or alcoholic beverages from starch-containing raw materials [17]. However, this yeast lacks the amylolytic activity necessary for starch utilization. To extend its substrate utilization range, the genes encoding glucoamylase (GA) were cloned into this organism [1, 6, 11, 28], including *Aspergillus awamori* glucoamylase [1,4;1,6-α-D-glucan glucohydrolase, EC 3.2.1.3] [17, 29]. Thus, *S. cerevisiae* has been used as a host organism for the production of several recombinant proteins [1, 10–12, 15, 26, 27]. Recently, Kilonzo et al. [17] used a recombinant *S. cerevisiae* strain C468/pGAC9 (ATCC 20690) containing the *A. awamori* glucoamylase gene. However, information regarding the production of recombinant proteins by this yeast strain is scarce in the current literature [29, 30].

In industrial production of recombinant proteins, a major concern is the plasmid instability of recombinant cells. The recombinants tend to lose their engineered characteristics due to the change or loss of plasmids. This is a major impediment in the scale-up of recombinant yeast strains. This is because *S. cerevisiae* multiplies by non-uniform budding, which leads to an uneven partitioning of the plasmids from mother to daughter cells. The formation of multiple buds compounds this problem, thereby leading to a high degree of segregational instability [13]. Thus, the problem of the probability of emergency of plasmid-free cells in *S. cerevisiae* is much higher than in the bacterium *E. coli* where binary fission ensures a better distribution of plasmids from the mother cell to the daughter cells. There are three types of plasmid instabilities: (1) Segregational instability arises from a defective system for partitioning of the plasmid DNA between the cells during cell division; (2) structural instability refers to changes in the plasmid DNA structure, such as deletion, insertion and rearrangement, and (3) competitive instability is attributed to the growth advantage of plasmid-free cells over plasmid-carrying cells, which usually results in the outgrowth of plasmid-carrying cells by plasmid-free cells under non-selective conditions [17, 22, 28]. The genetic instability combined with the effects of environmental factors causes recombinant cells to lose their plasmids during continuous or repeated batch culture processes. The loss of plasmid and the low growth rate of recombinant cells during the course of the production process could significantly reduce the production level of the desired protein product.

Both genetic factors (plasmid make-up, copy number, expression level, selective markers, genetic properties of the host) and environmental factors (media composition, dissolve oxygen tension, temperature, pH, dilution rate, bioreactor operation modes) affect plasmid stability in recombinant yeast. With increasing understanding of these factors, it is possible to enhance plasmid stability by manipulating plasmid composition and structure [23], changing the genetic and physiological properties of host cells [2], and manipulating the environmental conditions [17, 24, 25], dilution rate [13, 17, 29] and bioreactor operation modes [13, 15].

It has been observed that plasmid stability is significantly affected by the dilution rate in continuous cultures. Using selective media, [7] showed that their host-plasmid construct was stable at higher dilution rates. Similarly, the stability of the plasmid pJDB248 in *S. cerevisiae* in selective media was found to increase with dilution rate, while the plasmid stability in an auxotrophic mutant of *S. cerevisiae* containing a recombinant 2-μm plasmid increased substantially at a higher growth rate when grown in a selective medium. Moreover, studies showed that the stability of plasmid pYT760-ADH1 containing killer toxin cDNA in a chemostat culture of *S. cerevisiae* increased as a function dilution rate and reached 100% when the dilution rate was greater than a certain value when grown in a non-selective medium. In contrast, in non-selective media, the stability of the 2-μm-based pLG69Z plasmid studied in *S. cerevisiae* YN124 decreased with increasing dilution rate. These observations were supported by the work of Zhang et al. [29] who studied the expression of the *A. awamori* glucoamylase under the control of the yeast enolase I promoter in *S. cervisiae* in an air-lift bioreactor under non-selective conditions. However, Ibba et al. [16], while studying the expression of desulfotohirudin, a thrombin inhibitor, under the control of the constitutive glyceraldehyde-3-phosphate-dehydrogenase promoter, observed a low rate of plasmid loss at all dilution rates (0.06–0.17 h^{-1}) in non-selective medium. They also found that the effect of dilution rate on plasmid stability was insignificant when the protein expressed was well tolerated by *S. cerevisiase*. Recently, Gupter and Mukhergee [14] studied the stability of the shuttle plasmid Yep-51 containing *β*-galactosidase cDNA in continuous culture of *S. cerevisiae* AH22/Yep51 strain grown in a selective and non-selective media and found high and low plasmid stability at different dilution rates in selective and non-selective media, respectively.

It is evident from the above results that the effect of dilution rate on plasmid stability was related to the medium used. However, the precise effect of dilution rate on plasmid stability is still not clear.

In this work, the kinetics of glucoamylase production by recombinant *S. cerevisiae* C468/pGAC9 in a continuous airlift bioreactor was studied. A mathematical model was

developed and used to assess the nature of plasmid instability and to estimate the plasmid loss and growth kinetic parameters in a continuous mixed (plasmid-carrying and plasmid-free) cell culture. The effects of dilution rate on plasmid stability and glucoamylase production were also studied and reported here.

Materials and methods

Host strain and plasmid

The recombinant *S. cerevisiae* strain C468/pGAC9, which secretes glucoamylase into the extracellular medium [17, 29, 30], was employed in this study. The *S. cerevisiae* strain C468/pGAC9 (ATCC 20690) contains the hybrid plasmid vector pGAC9. The plasmid pGAC9 contains a portion of the yeast 2μ plasmid (2μ circle), a DNA fragment that encodes the *LEU* gene product (leucine), and a section of a glucoamylase gene from *A. awamori*, under control of the yeast enolase I promoter and terminator. The *S. cerevisae* host strain C468 (α *leu2-3 leu2–112 his311 his3-15 mal*$^-$) (ATCC 62995) is haploid, with auxotrophic markers for leucine and histidine, and carries mutation (*mal*$^-$) blocking the utilization of maltose as carbon source. Therefore, the host cell is complementary to the leucine prototrophy by inserting the selectable marker (*LEU* 2) into the expression plasmid, and the presence of the glucoamylase gene on the plasmid allows the host cell to grow on maltose [17].

Growth media

Two different media were utilized in this study. The selective YNBG medium contained 6.7 g l^{-1} yeast nitrogen base (YNB) without amino acids (Sigma), 0.04 g/l L-histidine (Sigma) and 20 g/l D-glucose. The stock slant culture was grown in the YNBG and maintained on YNBM containing 2% (w/v) maltose. The selective medium was used in preparing inocula for batch cultures and during bioreactor startup for continuous cultures. A complex non-selective YEPG medium contained 5 g/l yeast extract (Becton Dickinson), 10 g/l peptone (Becton Dickinson) and 20 g/l D-glucose. Without adjustment, the pH of these media was 5.0. For agar plates, the media also contained 2% (w/v) Fermtech-agar. The medium components other than D-glucose and maltose in YNBG, YEPG and YNBM, respectively, were sterilized by filtration (0.2 μm filter). D-Glucose and maltose were sterilized separately in an autoclave for 40 min at 121°C and 20 psi pressure. A selection procedure [29, 30] was applied to minimize any structural instability of the plasmid constructs during the preparation of the pre-culture.

Fermentation studies

Batch culture

Batch cultures were carried out in a custom-made stirred bioreactor with 1.2-l working volume operating in the batch mode, at 300 rpm and 30°C. They were inoculated with 60-ml flask culture grown overnight in YNB-G selective medium. The culture pH was monitored using an Ingold pH electrode and controlled at 5.0 by adding either 1.0 M NaOH or 1.0 M HCl acid. Foam control was achieved by the Silicone Antifoaming agent [0.3% (w/v)]. All experiments were conducted in triplicate. All chemicals were of analytical grade and were obtained from Sigma-Aldrich Co. Inc. (Oakville, ON, Canada). Fermentation samples were taken at regular time intervals to monitor the total cell concentration, D-glucose, starch, ethanol and glucoamylase concentrations and plasmid stability.

Continuous culture

Experiments were conducted in an inverse (annulus sparged) internal loop airlift fibrous-bed bioreactor (I-IL-AL-FBB). The schematic diagram of the experimental setup is shown in Figs. 1 and 2. The internal loop airlift-driven bioreactor containing a spiral-wound fibrous sheet material had a cylindrical column (I.D: 0.106 m) containing an inner concentric draft tube (downcomer) of 0.35 m in height and a diameter of 0.06 m. The bioreactor, with a total working volume of 9 l, was made of Plexiglas (polymethyl methacrylate) and surrounded by a water jacket for temperature control. The draft tube was positioned within the reactor column by stainless steel baffles and held vertically from the upper end using stainless steel rods. The bottom clearance h_b between the draft tube bottom and the base plate was kept constant at 0.059 m. The airlift bioreactor comprised of four sections: an expanded gas–liquid separator, a riser, a downcomer and a bottom section. The gas–liquid separator ($D_s = 0.257$ m), the riser (annulus) ($D_r = D_c - D_d = 0.032$ m) and the downcomer (draft tube) ($D_d = 0.060$ m) were circular in cross section. The riser-downcomer combination gave an A_r/A_d of 1.84 and a bioreactor volume of 9.0×10^{-3} m^3. The aspect ratio (L_d/D_c) of the bioreactor was 3.43. This ratio value was based on the downcomer height (neglecting the liquid level in the separator) [19–21].

The bioreactor was equipped with a dissolved (DO) probe and a pH electrode to monitor DO and pH, and then maintained at a constant temperature by circulating constant-temperature water through the bioreactor water jacket. When filtered sterile air was introduced into the bioreactor through the ring sparger, steady medium (liquid) circulation was generated throughout the bioreactor due to

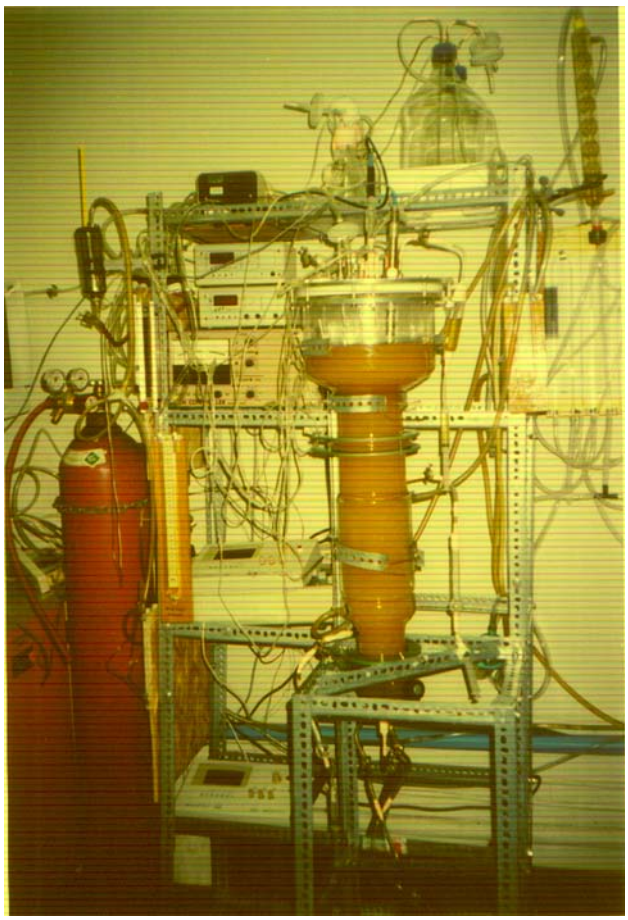

Fig. 1 Picture of the inverse internal loop airlift bioreactor

the bulk density difference between the riser and the downcomer (draft tube), providing mixing and nutrient transfer to the biomass. For a startup, the bioreactor was initially fed with the YNBG selective medium. The bioreactor was inoculated with 13.3% of a 48-h old culture grown in the selective medium. The culture was allowed to grow for ~20 h in the bioreactor.

After the cell density in the effluent had reached a steady state value, the fermentation was shifted to glucoamylase production phase by feeding the bioreactor with the rich non-selective YEPG medium at a selected dilution rate. Unless otherwise stated, the bioreactor was maintained at 30°C and aerated at a volumetric air flow rate of 9 l/min (i.e., 0.028 m/s, 1 vvm) through a system of packed glass fiber columns in series with a 0.2-μm pore filter. The dissolved oxygen tension in the bioreactor was maintained above 60% and monitored via dissolved oxygen electrode. The pH of the bioreactor was monitored using an Ingold pH electrode and controlled at 5.0 ± 0.05 by adding either 2.0 M NaOH or 2.0 M HCl acid. Foam control was achieved by the Silicone Antifoaming agent [0.3% (w/v)]. All experiments were conducted in duplicate.

To study the effect of dilution rate, the continuous cultures were operated at four different dilution rates, 0.05, 0.10, 0.20 and 0.37 1/h. Effluent samples from the bioreactor were taken at regular time intervals and assayed for the total cell concentration, D-glucose, ethanol and glucoamylase concentrations, and plasmid stability.

Analytical methods

Free cell concentration was measured by dry weight and optical density methods. The dry weight was determined by centrifuging 20 ml of sample in a 25-ml centrifuge tube at 5,000 rpm (1,500×g) to separate the cells from the spent fermentation broth. The clear supernatant was stored for use in the analytical stage. The cells were washed with deionized water twice and then dried overnight at 90–108°C. The optical density (OD) of the fermentation broth was measured at 600 nm (OD_{600}) in a 4-ml quartz cuvette using a UV/VIS spectrophotometer (752S, Micro Photonics Inc., Allentown, PA). Samples were diluted if the OD_{600} was above 0.3. The correlation between dry cell weight (N) and optical density (OD) was determined as $N = 1.6835$ OD ($R = 0.9986$, $R^2 = 0.9971$).

Glucose concentrations in the fermentation broth were determined using a glucose assay kit GAGO20-kiT (Sigma no. 027K8600). Samples from starch solutions were analyzed according to the method described by Kilonzo et al. [17] and Zhang et al. [29].

Ethanol concentration was determined by alcohol dehydrogenase enzyme according to the following equation [17]:

$$\text{Alcohol} + \text{NAD}^{+} \xrightarrow{\text{Alcoholdehydrogenase}} \text{Acetaldehyde} + \text{NADH} + \text{H}^{+}$$

and the method described in [17, 18]. Each reaction mixture (5 ml) contained 75 mM sodium PPi, 21 mM glycine, 75 mM semicarbazide HCl, 1.35 mM β-NAD, ethanol [0.005–0.03% (v/v)] and 0.12 mg of lyophilized alcohol dehydrogenase (Sigma, no. A7011). The pH of the reaction mixture was 8.7, and the reaction temperature was 30°C. The reaction was initiated by adding 0.1 ml of an appropriately diluted ethanol-containing sample to the reaction tube. After 12-min incubation, the reduced NADH produced was determined by measuring absorbance at 340 nm.

Glucoamylase activity was determined according to a modification of the assay described by Zhang [29, 30]. To 0.3 ml of enzyme solution, 0.5 ml of 1.5% soluble starch solution and 0.7 ml of 0.2 M acetate buffer (pH 5.0) was added and incubated at 37°C for 30 min. The reaction was stopped by adding 2.0 ml of 6 M H_2SO_4 into the reaction tube. The amount of reducing sugars (glucose) produced

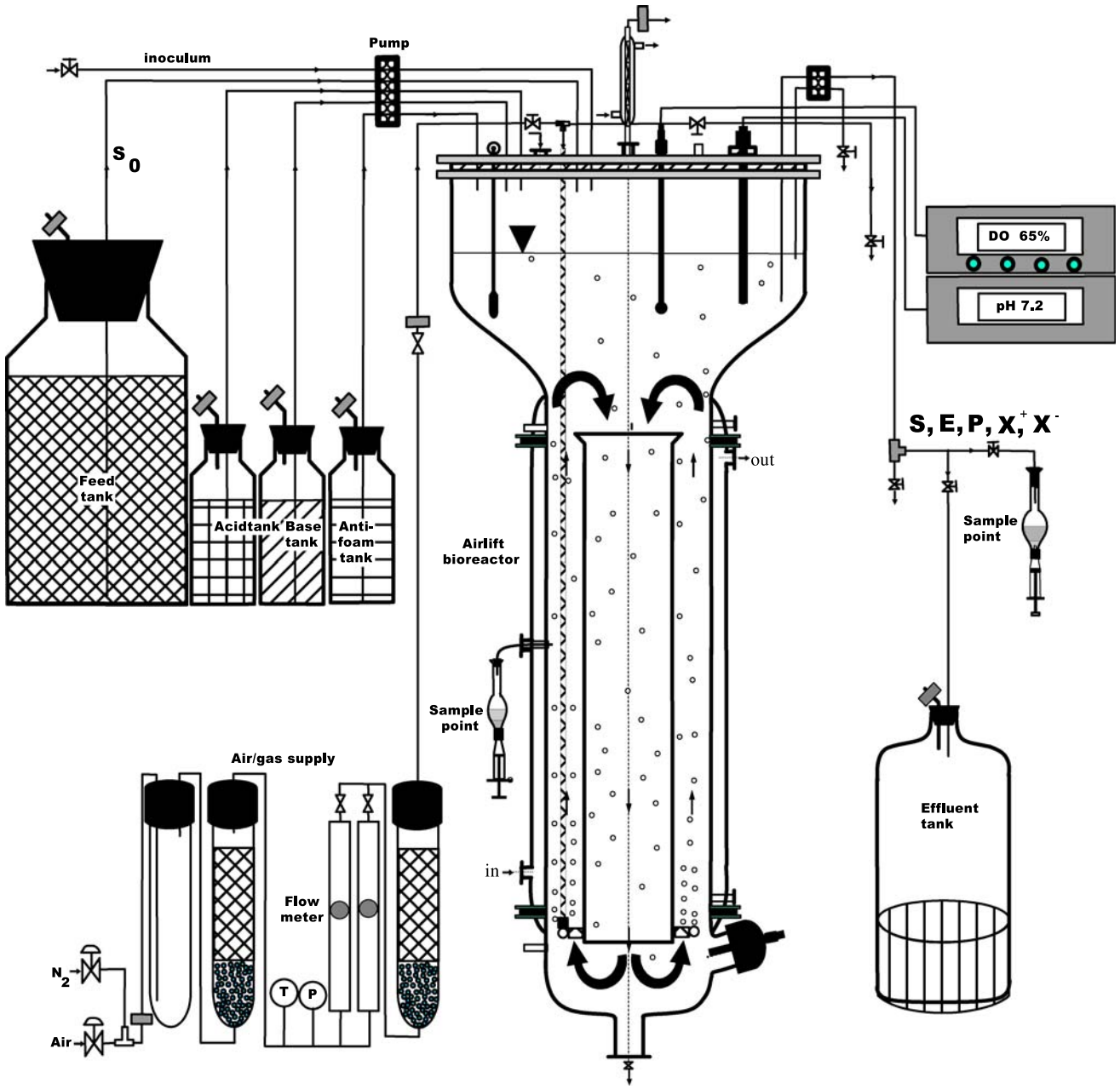

Fig. 2 Schematic diagram of the inverse internal loop airlift bioreactor setup for continuous recombinant yeast culture. Symbols: S_0 initial substrate concentration. S, E, P substrate, ethanol, and product concentrations. X^+ (or N^+), X^- (or N^-) plasmid-carrying and plasmid-free cell concentrations, respectively

during the reaction was assayed with the glucose kit (unless otherwise stated). One unit of glucoamylase activity is defined as the amount of enzyme in 1 ml to produce 1 μmol of equivalent glucose per minute from soluble starch in 0.2 M citrate buffer at pH 5.0 and 37°C [17].

The fraction of plasmid-harboring cells was measured by comparing the number of colonies grown on non-selective and on selective media plates. Samples from fermentation broth of actively growing cultures were diluted (1×10^{-5}–1×10^{-6}) to about 1,000 cell/ml using aseptic techniques. About 100 μl of this diluted (1×10^{-5}–1×10^{-6}) sample was spread onto both YNBG selective and YEPG non-selective agar plates (each five plates) [29, 30], and then incubated at 30°C for 2 days. All viable cells will multiply on YEPG non-selective agar plates, but only the plasmid–carrying cell can grow on YNB-D selective agar plates. The fraction of plasmid-carrying cells was found by counting the colony-forming units (CFU) on both types of plates (100–300 colonies per plate). All plate counts were taken from the average of at least five replicates.

Results and discussion

Kinetics of batch culture

Growth in YNBG selective medium

The kinetics of glucoamylase production from recombinant *S. cerevisiae* grown in the YNBG selective medium is shown in Fig. 3. Glucose was completely consumed for cell growth and ethanol production. During this period, no glucoamylase was produced because the ENOL 1 promoter was repressed by glucose. After glucose was depleted, ethanol was used for continued cell growth and glucoamylase production. As expected, the fraction of plasmid-carrying cells in the fermentation fluctuated around 93%. Consequently, the production of glucoamylase was significantly enhanced. This clearly indicated that the selective medium was important to the maintenance of the plasmid-stability and glucoamylase production. The cell yield resulting from growth on glucose was only 0.21 ± 0.02 and 0.02 ± 0.00 g/g for plasmid-carrying and plasmid-free cells, respectively.

It is noted that the glucoamylase production in this recombinant yeast is growth associated [4]. The glucoamylase is produced as an intracellular protein during the exponential growth phase. However, some proteases were found to be released to the medium in the stationary phase [3]. This is why the concentration of glucoamylase reduced under selective conditions (Fig. 3). The specific growth rate, yields and productivity for the culture grown on glucose are summarized in Table 1.

Growth in YEPG non-selective medium

The kinetics of glucoamylase production from recombinant *S. cerevisiae* C468/pGAC9 grown in the YEPG non-selective medium is shown in Fig. 4. Glucose was first consumed for cell growth and ethanol production. During this period, no glucoamylase was produced as previously stated above. After glucose was consumed, ethanol was utilized as the carbon source for continued cell growth, and a large amount of glucoamylase was produced in this period. Glucoamylase production in this batch culture was also growth-associated; the GA activity increased during the exponential growth phase, but remained nearly unchanged in the stationary phase. The fraction of plasmid-carrying cells continuously dropped to about 72%. The cell yield resulting from growth on glucose was 0.52 ± 0.13 and 0.08 ± 0.01 g/g for the plasmid-carrying and plasmid-free cells. The batch culture reached a maximum glucoamylase activity of 181 U/l.

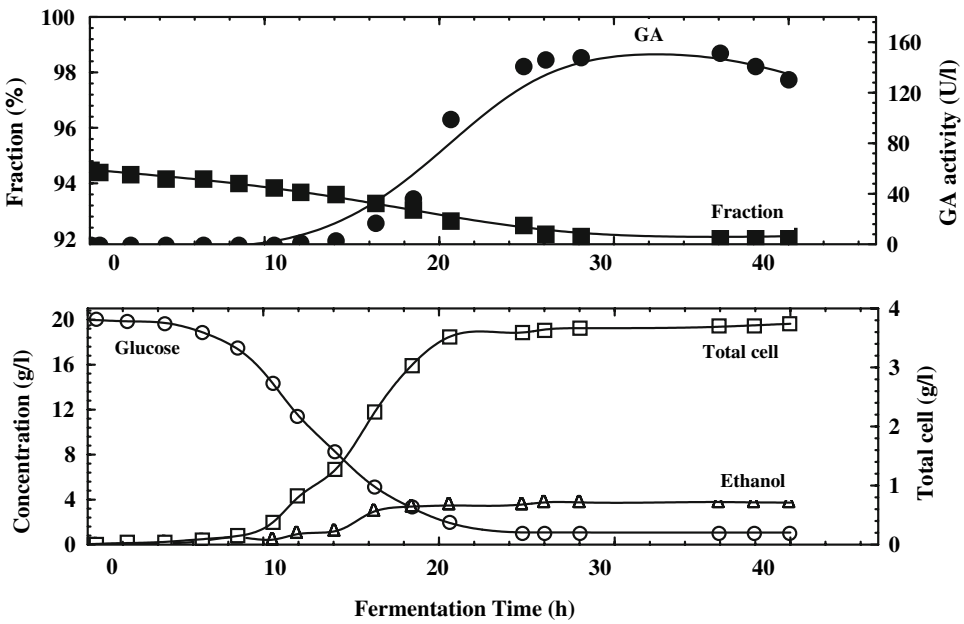

Fig. 3 Kinetics of GA (glucoamylase) production in batch grown in YNBG selective medium containing glucose as the carbon source. Fraction, the fraction of plasmid-carrying cells in the total population; EtOH, ethanol concentration; N^+, plasmid-carrying cells; N^-, plasmid-free cells

Table 1 Kinetics of glucoamylase production in batch cultures

Medium	μ_{max} (1/h)	$Y_{N/S}$ (g/g)	$Y_{P/N}$ (U/g)	$Y_{P/S}$ (U/g)	P (U/l/h)	P/N^+ (U/g/h)
Selective	0.19 ± 0.002	0.13 ± 0.002	37.2 ± 0.98	0.57 ± 0.07	5.41 ± 1.81	6.95 ± 0.03
Non-selective	0.377 ± 0.001	0.233 ± 0.110	46.1 ± 0.63	7.861 ± 3.05	6.62 ± 2.73	1.51 ± 1.512

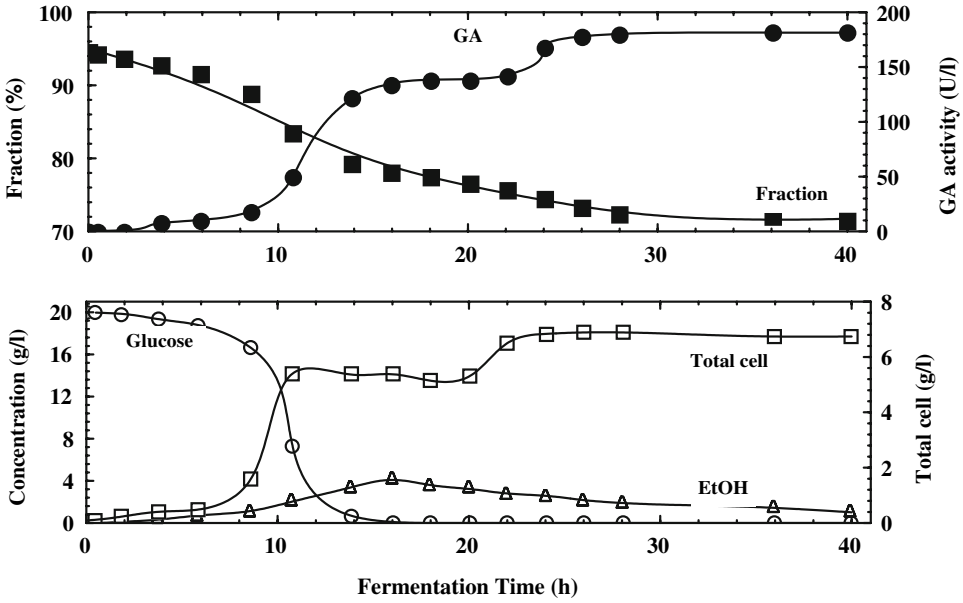

Fig. 4 Kinetics of GA (glucoamylase) production in batch grown in YEPG non-selective medium containing glucose as the carbon source. Fraction, the fraction of plasmid-carrying cells in the total population; EtOH, ethanol concentration; N^+, plasmid-carrying cells; N^-, plasmid-free cells

The comparisons of specific growth rate (μ), cell yield ($Y_{N/S}$), product yield ($Y_{P/S}$), specific GALase productivity ($Y_{P/N}$), volumetric productivity (P) and specific productivity (P/N^+) are given in Table 1. The specific productivity was determined from the volumetric productivity divided by the plasmid-carrying cell concentration, N^+.

As shown in Figs. 3 and 4, the fraction of plasmid-carrying cells dropped to a lower level at the end of the exponential phases, but then remained almost at the same level even when the glucose was depleted. This might be a result of the difference in the slow death rate between plasmid-carrying cells and plasmid-free cells (data not shown). The concentration of plasmid-carrying and plasmid-free cells in the cultures can be estimated from the total cell density and the fraction of plasmid-carrying cells in the culture. As indicated in Figs. 3 and 4, the concentration of plasmid-carrying cells is associated with the total cell concentration in batch culture and increases with the culturing period as compared to the plasmid-free cells (data not shown).

As can be seen in Table 1, faster growth and higher glucoamylase production were obtained with YEPG non-selective than with YNBG selective medium. Thus, for the subsequent continuous culture study, YNBG medium was used for the bioreactor startup and YEPG for glucoamylase production.

Kinetics of continuous culture

The time-course data of continuous cultures at four different dilution rates (0.05, 0.10, 0.20 and 0.37 h) are shown in Figs. 5, 6, 7 and 8, respectively. As shown in Figs. 5, 6,

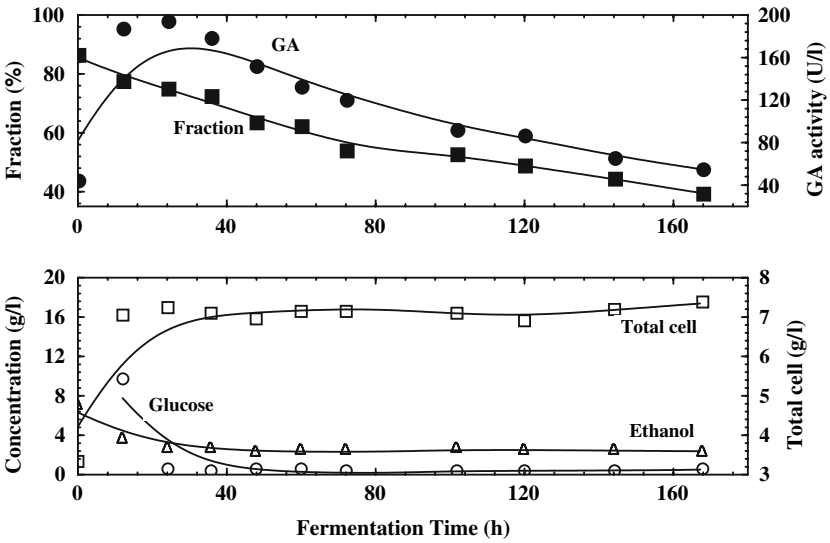

Fig. 5 GA production in continuous airlift bioreactor at 0.05 1/h dilution rate (bioreactor no. 1)

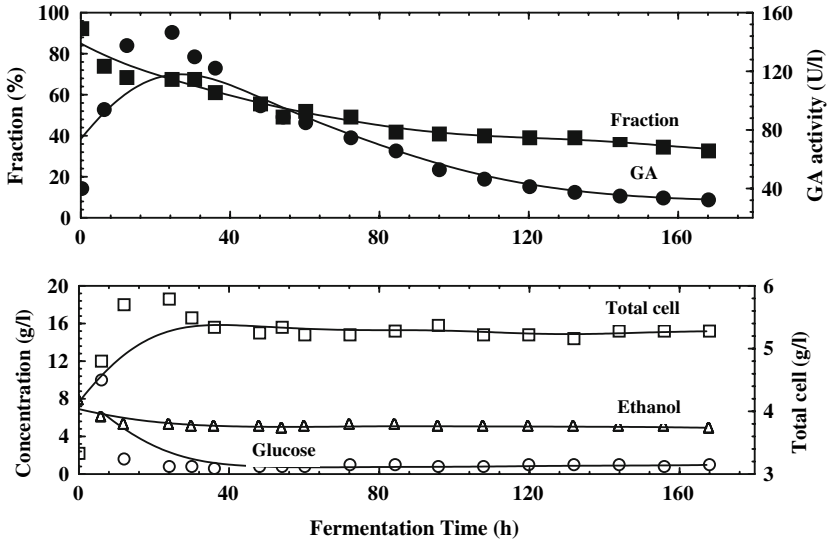

Fig. 6 GA production in continuous airlift bioreactor at 0.10 1/h dilution rate (bioreactor no. 3)

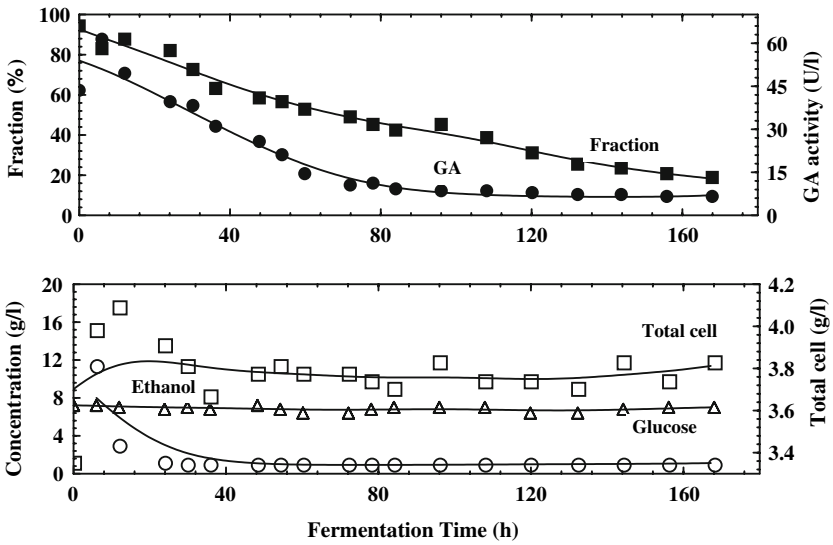

Fig. 7 GA production in continuous airlift bioreactor at 0.20 1/h dilution rate (bioreactor no. 4)

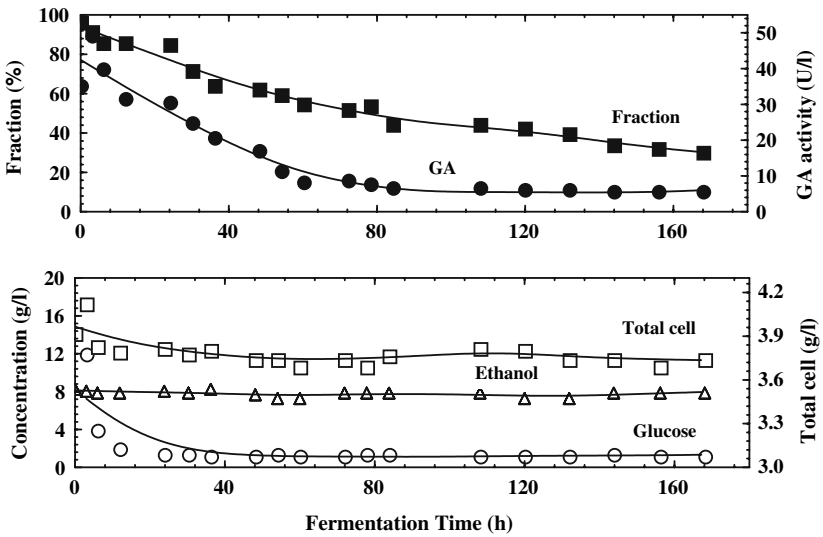

Fig. 8 GA production in continuous airlift bioreactor at 0.37 1/h dilution rate (bioreactor no. 6)

7 and 8, the bioreactors operating at dilutions rates <0.1 1/h reached relatively stable levels of cell density, glucose and ethanol concentrations within 40 h. However, the bioreactors remained stable throughout the culturing period for similar concentrations at dilutions >0.1 1/h (cf. Figs. 7 and 8). As expected, the fraction of plasmid-bearing cells continuously dropped to ~10% throughout the culturing period of ~120 h. In response to change from the selective medium to non-selective medium, glucoamylase (GA) production initially increased to a higher level. It then decreased gradually, following the occurrence of plasmid loss in the culture. However, the decrease in GA production in the bioreactor was generally slower than the plasmid loss rate. Also, at dilution rates <0.20 1/h the decline in GA production slowed down after 110–156 h of culturing time and leveled off after 50 h at higher dilution rates >0.2 1/h.

As also shown in Figs. 5, 6, 7 and 8, the specific productivity decreased at low dilution rates <0.10 h^{-1} (cf. Fig. 5) and increased significantly at dilution rates >0.10 1/h during the last ~86 h of the culturing period. The recombinant yeast used in this work had a reported low copy number [29]. Since specific productivity is usually proportional to the copy number, the unstable glucoamylase production could be attributed to the increase in the low plasmid copy number.

It is well known that plasmid copy number may vary with the cultural conditions and usually increases for cells grown at low growth (dilution) rate [29]. This explains why glucoamylase activity decreased at a slower rate than that of the fraction of plasmid-carrying cells. The increase in plasmid copy number might have resulted from the high generation numbers during continuous culture, since plasmid-carrying cells with fewer plasmid copy numbers at higher growth (dilution) rates >0.1 1/h should lose plasmids at a faster rate than those with high copy numbers at low growth (dilution) rates <0.1 1/h. Specific productivity (P/N^+) was calculated as total glucoamylase expression averaged for all plasmid-carrying cells with different copy numbers. After a certain number of generations, plasmid-carrying cells with fewer copy numbers were eliminated, and cells with high copy numbers were left in the culture, resulting in higher specific productivities (cf. Figs. 7, 8).

Effects of dilution rate

Effects of dilution rates on productivity

As the dilution rate was increased, the total cell density decreased from 6.90 ± 0.205 g/l at 0.05 1/h to 3.64 ± 0.023 g/l at 0.37 1/h; the average GA activity decreased from 86.62 ± 12.97 at 0.05 1/h to 26.39 ± 4.445 U/l at 0.37 1/h. The average ethanol concentration increased from 2.59 ± 0.124 g/l at 0.05 1/h to 8.90 ± 0.083 g/l at 0.37 1/h, and the residual glucose concentration increased from 2.08 ± 1.138 g/l at 0.05 1/h to 3.64 ± 1.425 g/l at 0.37 1/h. The effect of dilution rate on cell yield, product yield, specific GA production, volumetric productivity and specific productivity are shown in Table 2.

Effects of dilution rates on plasmid stability

The effect of the dilution rate on plasmid stability is shown in a plot of the fraction of plasmid-carrying cells as a function of the generation number (cf. Fig. 9). From Fig. 9, it can be seen that plasmid stability increases with increasing dilution rate. This is attributed to the fact that yeast divides with larger buds at faster growth rates, and the large buds increase the possibility of an even distribution of plasmids between the mother cell and the daughter cell [13, 18]. Also, it appears from Fig. 7 that the more the cells divide, the more likely they are to lose plasmids.

Comparison of cell yield ($Y_{N/S}$) and product yield ($Y_{P/S}$)

In general, cell yields and specific GA production from the continuous cultures were lower than those from the batch culture (cf. Table 1), but product yields were much higher for the continuous culture. The higher product yield in continuous culture was attributed to the higher fraction of plasmid-carrying cells (F^+) resulting from plasmid maintenance over the operation period. The lower cell yield in continuous culture could be attributed to the presence in the bioreactor of a large fraction of plasmid-free cells, which usually grew faster and had higher cell yields than plasmid-carrying cells in the batch culture. For continuous cultures,

Table 2 Kinetics of glucoamylase production in continuous cultures

Dilution rate D (1/h)	$Y_{N/S}$ (g/g)	$Y_{P/S}$ (U/g)	$Y_{P/N}$ (U/g)	P (U/l/h)	P/N^+ (U/g/h)
0.05	0.185 ± 0.065	2.716 ± 0.796	26.859 ± 8.199	3.974 ± 1.109	117.486 ± 29.056
0.10	0.324 ± 0.012	2.497 ± 0.521	7.962 ± 1.648	2.832 ± 1.394	23.067 ± 1.316
0.20	0.265 ± 0.060	2.506 ± 0.236	9.607 ± 0.824	1.789 ± 0.689	35.438 ± 1.766
0.37	0.133 ± 0.002	1.636 ± 0.082	2.176 ± 0.358	1.737 ± 0.756	44.422 ± 3.630

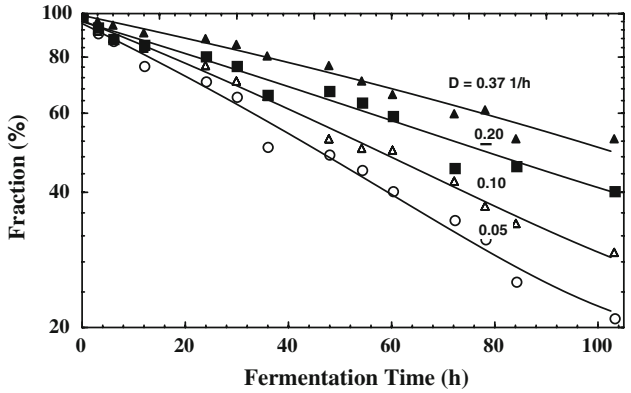

Fig. 9 Effect of dilution rate and generation number on plasmid stability

cell yield decreased from 0.338 ± 0.013 to 0.164 g/g, and product yield decreased from 9.267 ± 1.496 to 0.164 U/g as the dilution rate increased from 0.05 1/h to 0.37 1/h. The lower cell yield and higher product yield at 0.05 1/h might have resulted from changes in the bioreactor operating conditions.

Comparison of productivity (P) and specific productivity (P/N^+)

In general, the average bioreactor productivity from continuous culture was lower than the productivity from batch culture. As for continuous culture, productivity decreased with increasing dilution rate, from 3.97 ± 1.11 U/l/h at 0.05 1/h to 1.74 ± 0.78 U/l/h at 0.37 1/h. The productivity at 0.05 1/h was significantly higher at high dilution rates, possibly because of the influence from the environmental factors such as the pH. The specific productivity based on plasmid-carrying cells present in the continuous culture was several times higher than that in batch culture throughout the culturing period studied. A higher specific productivity at a higher dilution rate has also been reported for several other recombinant cell cultures [13, 14].

Kinetics and modeling of plasmid stability

Estimation of major genetic parameters and understanding their effects on productivity of recombinant microorganisms are essential to the design, control and optimization of large-scale recombinant cell culture processes. A mathematical model was developed to evaluate the kinetics of plasmid instability in a continuous culture. In this model, two important parameters (β and τ_0) are defined. The relative plasmid loss rate or probability of plasmid loss during each cell division, β, is equal to the specific production rate of plasmid-free cell, R, to the specific growth rate of plasmid-carrying cells, μ^+. β is indicative of segregational instability of the recombinant cell. The growth rate ratio between plasmid-carrying and plasmid-free cells, $\tau_0 = \mu^-/\mu^+$, is indicative of the selection between the N^+ and N^- population, whereas the ratio μ^-/μ^+ (growth rate ratio) is indicative of the competitive instability of the recombinant cell culture.

The model was developed based on the following assumptions:

1. The total number of cells N is constant after a few generations in the chemostat with constant operating conditions.
2. The dilution rate $D < 80\%$ of either μ^+_{max} or μ^-_{max}.

For a continuous culture, changes of the mixed cell populations, N^+, and N^-, with culturing time, t, can be described by the following two equations:

$$\frac{dN^+}{dt} = \mu^+ N^+ - \mu^+ \beta N^+ - DN^+ \quad (1)$$

$$\frac{dN^-}{dt} = \mu^- N^- + \mu^+ \beta N^+ - DN^- \quad (2)$$

The "cost" that plasmids impose on their host cells can be measured by the selection coefficient (also called selection advantage of plasmid-free cells) that is a relative growth rate difference

$$\alpha = 1 - \tau_0 \quad (3)$$

Note that $\alpha = 0$ if $\mu^+ = \mu^-$ and $\alpha = 1$ if $\mu^+ = 0$. Therefore, two parameters α and β according to this simple model represent the basic characteristics of plasmid-carrying cells (or the plasmid possessed by recombinant yeast).

We define two useful characteristics

$$M = \frac{N^-}{N^+} \quad (4)$$

$$F^+ = \frac{N^+}{N^+ + N^-} = \frac{1}{1+M} \quad (5)$$

where M is the ratio of the number of plasmid-free to plasmid-carrying cells, and F^+ is a fraction of plasmid-carrying cells in the yeast population. Then, using the definition of α and M we can rewrite the Eqs. (1) and (2) in two forms:

$$\frac{dM}{dt} = \mu^+ \left[\frac{\alpha}{1-\alpha} M + (1+M)\beta\right] \quad (6)$$

$$\frac{dM}{dt} = \mu^- [\alpha M + \beta(1-\alpha)(1+M)] \quad (7)$$

In general, equations (6) and (7) cannot be solved analytically because of the dependence of α, μ^- and μ^+ on the substrate concentration.

Table 3 Estimated values of instability parameters during continuous culture or recombinant *Saccharomyces cerevisiae* C468/pGAC9 in a continuous stirred tank bioreactor

Parameter	D (1/h)			
	0.05	0.10	0.20	0.37
β (−)	0.059 ± 0.001	0.085 ± 0.005	0.043 ± 0.002	0.020 ± 0.006
α (−)	0.135 ± 0.022	0.284 ± 0.031	0.191 ± 0.013	0.177 ± 0.051
μ^+ (1/h)	0.047 ± 0.007	0.042 ± 0.004	0.046 ± 0.001	0.047 ± 0.007
μ^- (1/h)	0.053 ± 0.002	0.059 ± 0.001	0.057 ± 0.005	0.057 ± 0.004
τ_0 (−)	0.865 ± 0.003	0.716 ± 0.011	0.809 ± 0.008	0.823 ± 0.003

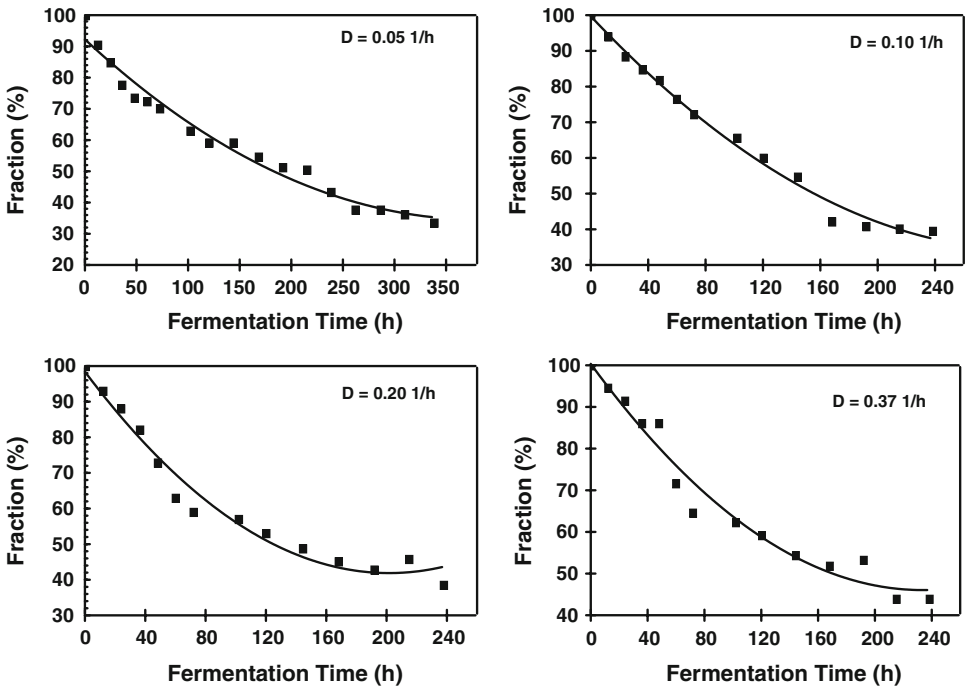

Fig. 10 Comparison of model simulation and data on the fraction of plasmid-carrying cell population in continuous cultures at various dilution rates: (**a**) 0.05 1/h; (**b**) 0.10 1/h; (**c**) 0.20 1/h; (**d**) 0.37 1/h (*curves* show model simulation and *symbols* show experimental data)

Estimating a probability of plasmid loss β and selection coefficient α

The general approach applied to estimate α and β at the approximation $\alpha = \text{constant}$ has been done by assuming a constant steady-state in chemostat with $\mu^+ = D$ [5, 9]. Rewriting Eq. 6 by replacing M with F^+ we obtain

$$\frac{dF^+}{dt} = -\mu^+ F^+ \left[\frac{\alpha}{1-\alpha}(1-F^+) + \beta\right] \tag{8}$$

Assuming that $\mu^+ = D$ and $\alpha = const$ we find its explicit solution

$$F^+ = \frac{F_0^+[\beta(1-\alpha)+\alpha]}{\alpha F_0^+ + [\beta(1-\alpha)+(1-F_0^+)\alpha]2^{\lambda\left[\frac{\alpha}{1-\alpha}+\beta\right]}} \tag{9}$$

where

$$\lambda = \frac{\mu^+ t}{\ln 2} = \frac{Dt}{\ln 2} \tag{10}$$

(Davidson and Dunn [5, 9]). A more elegant approach to estimate α and β if $\alpha = const$ has been previously developed at the approximation $\beta = 0$ by Duetz and van Andel [8]. Their major observation was that after a short phase of expansion of plasmid-carrying cells in the chemostat, the fraction of plasmid-carrying cells obeys the equality

$$\mu^+ F^+ + \mu^-(1-F^+) = D \tag{11}$$

The growth rate of plasmid-carrying cells can be then determined explicitly:

$$\mu^+ = \frac{D(1-\alpha)}{1-\alpha F^+} \tag{12}$$

Replacing μ^+ in Eq. 6 with Eq. 12, assuming $\alpha = const$, and integrating we obtain

$$\left[\frac{\alpha(1-F^+)+\beta(1-\alpha)}{\alpha(1-F_0^+)+\beta(1-\alpha)}\right]^{(1-\beta)(1-\alpha)} = \left(\frac{F^+}{F_0^+}\right)2^{\lambda[\alpha+\beta(1-\alpha)]} \tag{13}$$

where

$$\lambda = \frac{Dt}{\ln 2} \tag{14}$$

By fitting solution (13) to the experimental data, one can estimate the parameter β ($\alpha = 0$, $\mu^+ = \mu^-$) and α ($\beta = 0$) as a function of dilution rate (D) and F^+. The results of the fitting are summarized in Table 3. As shown in Table 3, the relative plasmid loss β decreased with increasing dilution rate. The selection coefficient decreased also with dilution up to $D = 0.20$ 1/h and then starts to increase as it approaches the washout point ($D = 0.40$ 1/h). Equations 11 and 12 can be used to estimate the specific growth rate of plasmid-free cells (μ^-) and the specific growth rate of plasmid-carrying cells (μ^+) at different dilution rates using the pre-determined values of the model parameters (β and α).

The model thus suggested that plasmid instability in the recombinant *S. cerevisiae* culture studied was caused mainly by the segregational instability instead of competition instability. The results from the model were consistent with the experimental results shown in Fig. 9 that plasmid stability in continuous culture increased with dilution rate. As shown in Fig. 10, the simulation data were slightly higher than the experimental data (cf. Figs. 5, 6, 7, 8) at low dilution rates. However, the model simulates the experimental data well at higher dilution rates.

Discussion

Compared to batch culture, continuous culture improved the productivity of recombinant glucoamylase in the chemostat. The specific productivity of plasmid-carrying cells increased with time at higher dilution rates in the continuous culture, possibly because of natural selection of high-copy number cells over the long operation period. The plasmid stability, glucoamylase production and concentration of plasmid-carrying cells were associated with growth (dilution) rate and followed first-order kinetics, which was consistent with experimental results. The plasmid stability was higher at high dilution rates, while glucoamylase production was lower at higher dilution rates. A model was developed for estimating the growth difference between plasmid-carrying and plasmid-free cell, generation rate of plasmid-free cells, specific growth rates of plasmid-carrying and plasmid-free cell and the probability of plasmid loss. The probability of plasmid loss decreased, while specific growth rates increased with dilution rates. The model can be used to assess the nature of plasmid instability in a recombinant yeast continuous culture. In the present case, segregational instability was dominant.

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