

Effect of Medium Composition on Glucoamylase Production During Batch Fermentation of Recombinant *Saccharomyces cerevisiae*

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ABSTRACT

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Plasmid stability of the recombinant *Saccharomyces cerevisiae* C468/pGAC9 (ATCC 20690) strain harboring a pGAC9 plasmid with glucoamylase genes has been investigated in shake flasks and in a bioreactor system using various compositions of media containing glucose or starch as the main carbon and energy source. The medium composition affected both the growth characteristics of *S. cerevisiae* and stability of the plasmid. Superior plasmid stability was obtained in yeast minimal medium and in complex medium with 0.5 to 2% D-glucose. Plasmid stability of 92% was obtained in complex medium with 2% D-glucose yielding 48 units of glucoamylase/g of cells compared to 54% plasmid stability achieved with 2% soluble starch, which yielded 23 units of glucoamylase/g of cells. The plasmid stability increased at high growth rates and decreased with increasing starch concentration in the complex media as compared to glucose medium. The kinetic characteristics of biomass and glucoamylase production were investigated, and a growth kinetic model was used to interpret the experimental results.

Key words: glucoamylase, kinetic models, plasmid stability, recombinant yeast, *Saccharomyces cerevisiae*.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* has been used to express a range of heterologous proteins of pharmaceutical and industrial importance^{1,6}. The importance of this organism lies in the fact that it is not affected by pyrogens (as in *E. coli*) or oncogens or viruses (as in mammalian systems) thus making it a safe organism for humans¹⁸. *S. cerevisiae* is also widely used for commercial production of ethanol or alcoholic beverages from starch-containing raw materials³. *S. cerevisiae*, however, lacks amyolytic activity necessary for starch utilization. To extend its substrate utilization range, the genes encoding glucoamylase (GA) were cloned into this organism^{1,3,5,6,9,13,18}, including

Aspergillus awamori glucoamylase [1,4;1,6- α -D-glucan glucohydrolase, EC 3.2.1.3]^{3,9,13}. However, *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 instability⁷. Thus, the problem of 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.

The genetic stability of a plasmid is dependent on the characteristics of the host and plasmid⁷, as well on the growth environment^{14,15}. Environmental factors considered at the fermentation level included operating strategy and medium composition. Medium compositions affect the stability of the plasmid through different metabolic pathways and regulatory systems of the host. Nunberg et al.¹³ prepared different constructs of the strain C468, including strains C468/pAC1, C468/pGAC9, C468/pGC21, while Cole et al.³ prepared the following constructs: C468/YEpMAC101, C468/YEpGAC9, C468/YEpPM16, 162/YIpPM20-2, and C468/YEpPM18. The recombinant *S. cerevisiae* strains C162/YIpPM20-2 and C468/YEpPM18, which secrete glucoamylase were found to have higher ethanol productivity associated with superior ability to utilize the hydrolysis products of starch when compared with C468/pGAC9, which is also a glucoamylase secretor. The ethanol production of these strains increased with increasing starch concentration, leading to higher levels of reducing sugar in the medium. All three strains were found to be stable in YEP-G (containing 2% D-glucose), exhibiting plasmid stability higher than 70%^{3,19,20}. However, results of Cole et al.³ shake flask experiments indicated increased instability of C468/pGAC9 in media containing higher concentrations of starch^{3,7,13}. The lower growth rates of this strain are thought to be due to a defect in the glucoamylase domain to act on the branched natural substrates, possibly related to a problem in the secretion mechanism. Furthermore, high plasmid instabilities associated with low growth rates of C468/pGAC9 in media containing higher starch concentrations could be responsible for the low levels of production in shake flask experiments. Control experiments in a batch fermenter also showed that this strain could produce very little ethanol in

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Table I. Medium type and composition used in this study.

Medium type	Amount per litre of distilled H ₂ O
YNB-G	YNB (yeast nitrogen base) without amino acids, 6.7 g; (NH ₄) ₂ SO ₄ , 5 g; L-histidine, 0.04 g; D-glucose, 5 to 50 g
YNB-M	YNB (yeast nitrogen base) without amino acids, 6.7 g; (NH ₄) ₂ SO ₄ , 5 g; L-histidine, 0.04 g; maltose, 20 g
YEP-G	Yeast extract, 5 g; peptone, 10 g; L-histidine, 0.04 g; D-glucose, 5 to 50 g
YEP-Ss	Yeast extract, 5 g; peptone, 10 g; L-histidine, 0.04 g; soluble starch, 50 g
YEPG	Yeast extract, 10 g; peptone, 20 g; L-histidine, 0.04 g; D-glucose, 50 g
YEPSs	Yeast extract, 10 g; peptone, 20 g; L-histidine, 0.04 g; soluble starch, 5 to 30 g
YNB-G-agar	2% (w/v) of agar in YNB-G
YEP-G-agar	2% (w/v) of agar in YEP-G
YNB-M-agar	2% (w/v) of agar in YNB-M

media containing 2% of different types of starch (“washed” starch, cassava starch, and potato starch)¹³. Medium composition is also important for the production of secreted proteins as substances present in the fermentation medium may reduce the efficiency of subsequent purification steps. Although continuous experiments are common in plasmid stability studies, our main objective in conducting batch experiments was to study the behavior of plasmid loss in such fermentations.

In this study, the recombinant yeast, *S. cerevisiae* strain C468 carrying the plasmid pGAC9 for the production of fungal glucoamylase was used as a model system. In the first part of this study, the segregational instability of the plasmid pGAC9 in various media was studied. The effects of medium composition on segregation instability, secretion of extracellular glucoamylase and biomass growth were examined in batch cultures both in shake flasks and in a bioreactor. The second part of this study was focused on the kinetics of biomass growth and glucoamylase production of batch cultures of recombinant *S. cerevisiae* C468/pGAC9. Linear and non-linear unstructured kinetic models available in the current literature^{2,11,12} were tested. The detailed mathematical analysis of growth and glucoamylase production kinetics of *S. cerevisiae* C468/pGAC9 presented in this study forms a basis for further modeling studies.

MATERIALS AND METHODS

Recombinant yeast strain and plasmid

The recombinant *Saccharomyces cerevisiae* strain C468/pGAC9, which secretes glucoamylase into the extracellular medium^{13,19,20} was employed in this study. The *Saccharomyces cerevisiae* strain C468/ pGAC9 (ATCC 20690) contains the hybrid plasmid vector pGAC9. The plasmid pGAC9 contains a portion of the yeast 2 μ plasmid (2 μ micron circle), a DNA fragment which encodes the *LEU* gene product (leucine), and a section of a glucoamylase gene from *Aspergillus awamori*, under control of the yeast enolase I promoter and terminator. The *S. cerevisiae* 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 maltose¹³.

Growth media and conditions

The different media utilized in this study are listed in Table I. The medium components other than D-glucose, maltose, and starch in YNB-G, YNB-M, YEP-G, and YEP-Ss were sterilized by filtration (0.2 μ m filter). D-glucose, maltose, and starch were sterilized separately for 20 min at 121°C. The stock slant culture was grown in YNB-G containing 2% (w/v) D-glucose and maintained on YNB-M containing 2% (w/v) maltose. The pre-cultures were grown in YNB-G containing 2% (w/v) D-glucose and were used as an inoculum on a 2% (v/v) basis. A selection procedure^{19,20} was applied to minimize any structural instability of the plasmid constructs during the preparation of the pre-culture.

Fermentation studies

Two sets of experiments were conducted for investigating the effect of medium composition on plasmid stability of YNB-G, YEP-G, YEPG, YEP-Ss, and YEP-Ss. In the first set, batch cultures were performed in Erlenmeyer flasks. Cells from a single colony were used to inoculate a 250-mL seed culture flask containing 50 mL of YNB-G selective medium. After 24 h, a 500-mL flask containing 300 mL (YNB-G, YEP-G) medium was inoculated (1% (v/v)) and incubated at 30°C in a Series 25 reciprocating (environmentally controlled) incubator shaker (New Brunswick Science, Co. Inc., NJ) at 200 rpm. The pH followed its natural course.

In the second set of experiments, the cells were cultivated in a custom made stirred bioreactor using YEP-G medium. The bioreactor had a working volume of 1.2 L and operated in a batch mode at 300 rpm and 30°C. The culture pH was monitored using an Ingold pH electrode and controlled at pH 5.0 by adding either 1.0 M NaOH or 1.0 M HCl acid. Foam control was achieved by the use of a 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.

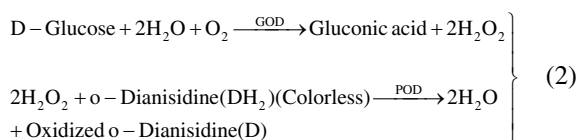
Analytical methods

Free cell concentration was measured by dry weight and optical density methods. The dry weight was determined by centrifuging 5 mL of the flask samples or 20 mL of the bioreactor sample in a 25 mL centrifuge tube at 5000 rpm (1500 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 (OD₆₀₀) in a 4-mL quartz cuvette using a UV/VIS spectrophotometer (752S, Micro Photonics Inc., Allentown PA). Samples were diluted if the OD₆₀₀ was above 0.3. The optical densities (OD) were converted to dry cell weight (X) using the correlation (R = 0.99, R² = 0.99):

$$X(\text{dry cell weight, g/L}) = -0.004 + 1.855\text{OD}_{600\text{nm}} \quad (1)$$

The glucose concentration was determined using glucose kit (GAGO20-Kit) from Sigma-Aldrich, Inc. (St. Louis, MO). The procedure was based upon the following coupled enzymatic reactions:



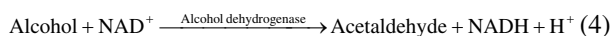
The amount of dye formed from DH₂ is a measure of the glucose oxidized. The intensity of the violet color measured at 570 nm (535-585 nm range) is related to that of a glucose standard:

$$\text{Glucose}[\text{mg/L}] = \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{std}}} * 50 \quad (3)$$

D-Glucose concentrations in the fermentation broth were determined using a glucose assay kit GAGO20-kiT (Sigma No. 027K8600).

Starch was measured colorimetrically. Known volumes of sample aliquots were mixed with 5 µL of iodine solution (0.5% KI and 0.15% I₂). These samples were diluted to a final volume of 15 µL with dH₂O. The optical density was read at 550 nm against the blank containing 5 µL iodine solution and 10 µL of dH₂O.

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



described elsewhere^{10,17}. 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 to 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 35°C. The reaction was initiated by adding 0.4 ml of an appropriately diluted ethanol-containing sample to the reaction tube. After 25-min incubation, the reduced NADH produced was determined by measuring absorbance (OD) at 340 nm before (OD₁) and after (OD₂) addition of the alcohol dehydrogenase (ADH) enzyme and the incubation period. Prior deproteinization of fermentation fluid by perchloric acid was not required for measuring ethanol. A standard curve was made for each set of assays performed (unless stated otherwise). OD₁ – OD₂ = <(OD) was calculated using the equation:

$$E = \frac{\Delta(\text{OD})}{\epsilon d} \quad (5)$$

where ε = absorbance coefficient (cm²/µmole), OD =

absorbance, <(OD) = absorbance change, d = light path (cm). The ethanol concentration E of the sample was given by:

$$E(\mu\text{g/mL}) = 122.9\Delta(\text{OD}) \quad (6)$$

Glucoamylase activity was determined according to a modification of the assay described by Zhang et al.^{19,20} 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) were added and incubated at 37°C for 30 min. The reaction was stopped by adding 2.0 mL of 12 N H₂SO₄ into the reaction tube. The amount of reducing sugars (glucose) produced 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.

The fraction of plasmid-bearing 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 1000 cells/mL using aseptic techniques. About 100 µL of this diluted sample was spread onto both YNB-G selective and YEP-G non-selective agar plates (five plates each)^{18,19}, and then incubated at 30°C for 2 days. All viable cells will multiply on YEP-G non-selective agar plates, but only the plasmid-carrying cell can grow on YNB-G selective agar plates. The plasmid segregational stability, i.e., the fraction of plasmid-containing cells (F⁺) in the cultures was calculated as the ratio of the average number of colony-forming units (CFU) on the YNB-G selective agar plates to the total number of colonies on the YEP-G non-selective agar plates according to the equation:

$$F^+ = \frac{\Sigma\text{CC}_{\text{Selective}}}{\Sigma\text{CC}_{\text{Non-selective}}} \times 100\% \quad (7)$$

where ΣCC_{selective} and ΣCC_{Non-selective} are summation of the counts of colonies on the selective and non-selective media, respectively. All plate counts were taken from the average of at least five replicates.

Segregational loss rates (S_L) over time t generations were calculated from Eq. 1, where F_t⁺, F₀⁺ are the proportions of plasmid-containing cells at time = t and time t = 0 respectively. This parameter describes the overall loss rate¹⁵.

$$S_L = 2 \left[1 - \exp \left(\frac{\ln \left(\frac{F_t^+}{F_0^+} \right)}{t} \right) \right] \quad (8)$$

The maximum specific growth rate, μ_{max}, was calculated from least-squares linear regression equation:

$$\mu_{\text{max}} = \frac{\ln X - \ln X_0}{t - t_0} \quad (9)$$

where X and X₀ are the final and initial total cell concentrations; t and t₀ are the final and initial time, respectively.

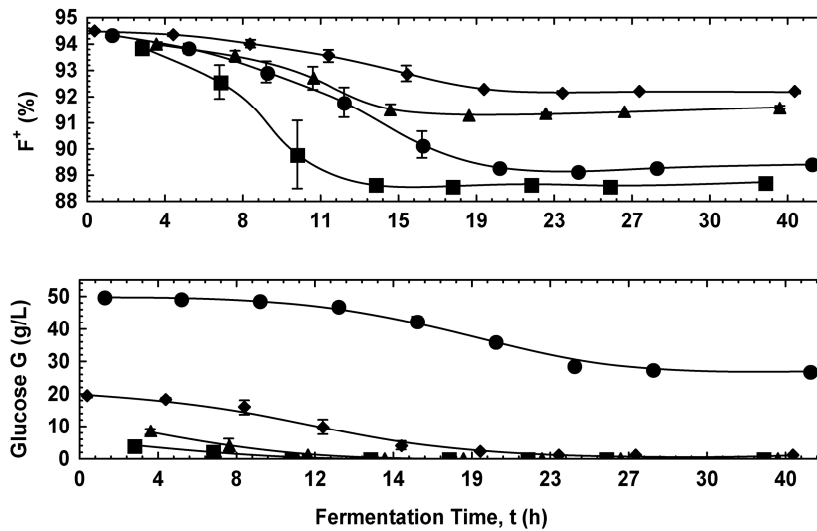


Fig. 1. Fermentation characteristics of *S. cerevisiae* C468/pGAC9 grown in shake flasks in YNB-G medium. Time profile of glucose concentration and plasmid stability (F^+) in YNB-G medium containing (■) 5, (▲) 10, (◆) 20, and (●) 50 g/L initial D-glucose (G_0) concentration.

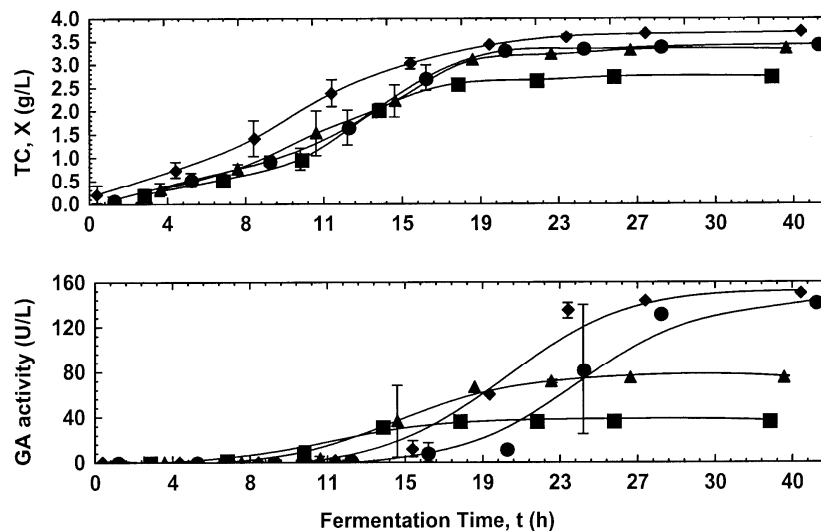


Fig. 2. Fermentation characteristics of *S. cerevisiae* C468/pGAC9 grown in shake flasks in YNB-G medium. Time profile of total cell (TC) (X) concentration and glucoamylase (GA) activity in YNB-G medium containing (■) 5, (▲) 10, (◆) 20, and (●) 50 g/L initial D-glucose (G_0) concentration.

RESULTS AND DISCUSSION

Several batch fermentations in Erlenmeyer flasks and in a custom made stirred tank bioreactor were carried out with plasmid-bearing cells using selective and non-selective media.

Shake flask experiments

The effect of carbon source in different medium types on cell growth, plasmid stability and protein secretion of C468/pGAC9 strain was examined. The C468/pGAC9 strain was initially grown either in YNB-G selective medium or in complex media (YEP-G, YEPG, YEP-Ss, and

YEPSs) (Table I) until the cells entered the stationary phase. Typical time courses of dry cell mass, residual D-glucose concentration, plasmid stability, and glucoamylase activity of the recombinant yeast cell culture containing different initial concentrations of D-glucose are presented in Figs. 1–2 for the YNB-G medium. The D-glucose was completely exhausted when this medium contained 5 or 10 g/L.

A dramatic effect on the recombinant yeast fermentation resulted when complex non-selective media YEP-G or YEPG were utilized. Figs. 3–5 show the time profile of dry cell mass, residual D-glucose concentration, plasmid stability, and glucoamylase activity of the recombinant

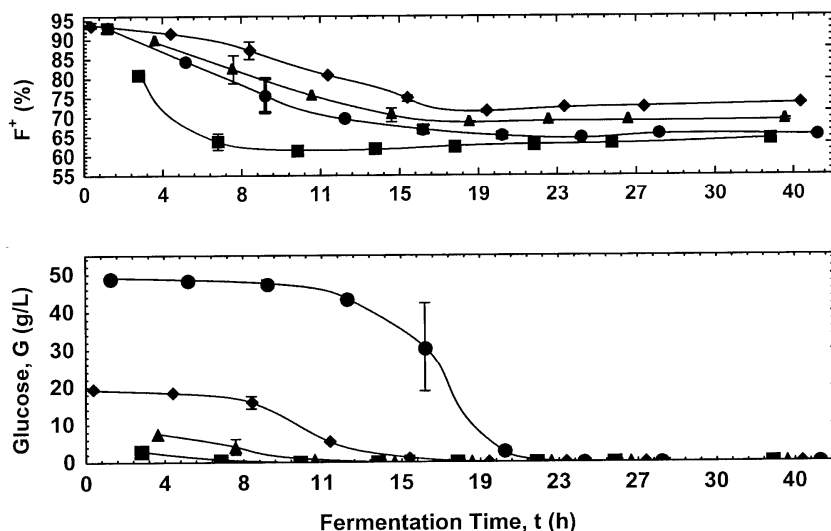


Fig. 3. Fermentation characteristics of *S. cerevisiae* C468/pGAC9 grown in shake flasks in YEP-G medium. Time profile of glucose (G) concentration and plasmid stability (F^+) in YEP-G medium containing (■) 5, (▲) 10, (◆) 20, and (●) 50 g/L initial D-glucose (G_0) concentration.

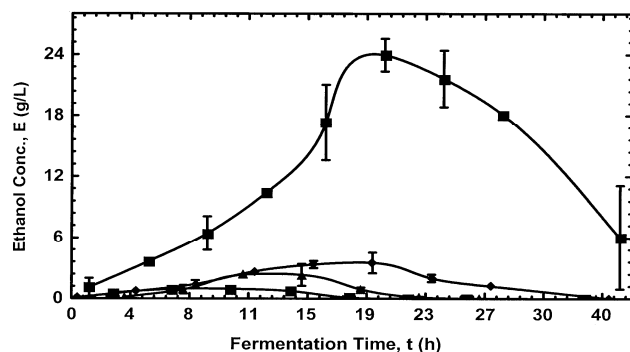


Fig. 4. Fermentation characteristics of *S. cerevisiae* C468/pGAC9 grown in shake flasks in YEP-G medium. Time profile of ethanol (E) concentration in YEP-G medium containing (■) 5, (▲) 10, (◆) 20, and (●) 50 g/L initial D-glucose (G_0) concentration.

yeast cell culture containing different initial concentrations of D-glucose for the YEP-G medium. The cells consumed all the D-glucose, as opposed to the yeast minimal selective medium YNB-G. The recombinant yeast also underwent a diauxic growth, first consuming D-glucose ($-r_G = 3.88$ g/L/h; Appendix A) during the first growth phase as the carbon source for glucoamylase, cells, CO_2 and ethanol production [$-r_E = 1.54$ g/L/h; Appendix A]. Secondly, when glucose was depleted in the medium, the ethanol produced during the first growth phase was utilized ($-r_E = 1.54$ g/L/h) as the carbon source in the second growth phase to form more glucoamylase, cells and CO_2 . The glucoamylase production was growth associated as well. These findings agree well with results reported for glucoamylase production with other recombinant yeast strains^{4,6}.

One interesting phenomenon found in these batch fermentations was that the fraction of plasmid-carrying cells (F^+) 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 (X^+) and plasmid-free cells (X^-).

Furthermore, when starch was used as the carbon source in complex media (YEP-Ss and YEPSSs), only about 40% of the starch could be hydrolyzed by the recombinant yeast. Figs. 6–8 show the time profile of dry cell mass, residual D-glucose concentration, plasmid stability, and glucoamylase activity of the recombinant yeast cell cultures containing different initial concentrations of soluble starch for the YEP-Ss medium.

Similar trends were observed in the time profiles of dry cell weights. Dry cell weights determined in the stationary phase of growth were found to increase with increasing initial D-glucose concentrations up to 20 g/L, and then leveled off in YNB-G medium after 25 h of the fermentation period. The cell mass increased by a factor of 1.31 when the initial D-glucose concentration was changed from 5 to 20 g/L. The remarkable decrease in the biomass yield (Table II) in YNB-G medium suggested a threshold value for D-glucose between 10 g/L and 20 g/L above which the cells cannot grow and utilize D-glucose further. The use of higher D-glucose concentrations (> 20 g/L) did not result in any appreciable increase in the final cell mass which reached 3.23 g/L in YNB-G medium. Low levels of biomass yield associated with high concentrations of initial D-glucose in YEP-G medium (0.057 g/g) suggest a metabolic pathway in the direction of ethanol production rather than respiration to generate more biomass¹⁶. This is partly attributed to Crabtree effect of glucose (i.e. the change in catabolism from glucose oxidation to glucose fermentation at glucose concentrations greater than 0.1 g/L). Fig. 9 shows a simplified representation of the major catabolic pathways in the recombinant *S. cerevisiae* yeast. When the glucose concentration in the YEP-G/YEPG media was too large ($\gg 0.1$ g/L) for the yeast cell's oxidative capacity, excess glucose was fermented to

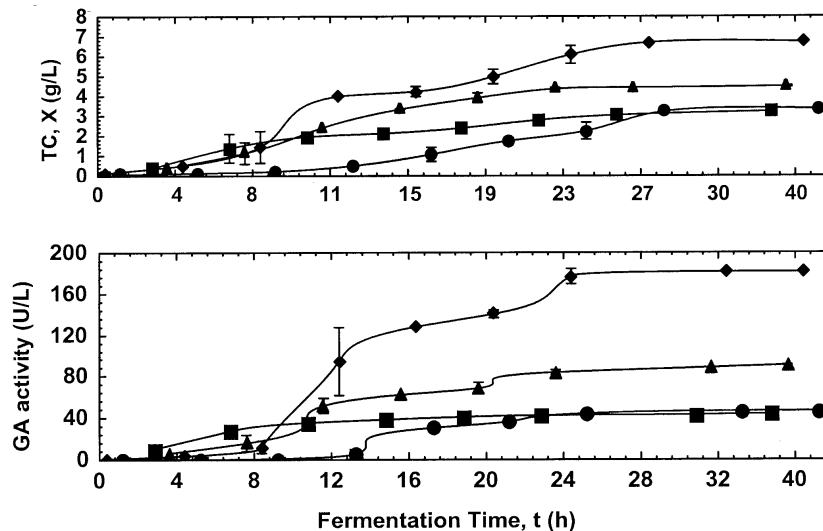


Fig. 5. Fermentation characteristics of *S. cerevisiae* C468/pGAC9 grown in shake flasks in YEP-G medium. Time profile of total cell (TC) (X) concentration and glucoamylase (GA) activity in YEP-G medium containing (■) 5, (▲) 10, (◆) 20, and (●) 50 g/L initial D-glucose (G_0) concentration.

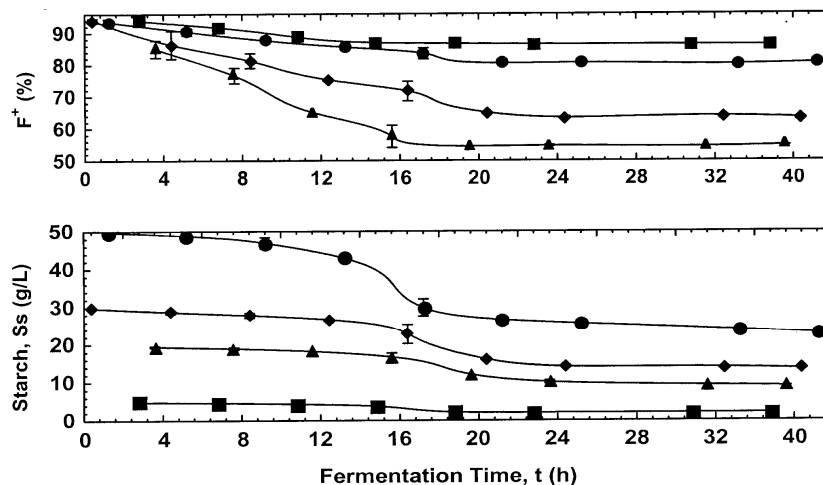


Fig. 6. Fermentation characteristics of *S. cerevisiae* C468/pGAC9 grown in shake flasks in YEP-Ss medium. Time profile of soluble starch (Ss) concentration and plasmid stability (F^+) in YEP-Ss medium containing (■) 5, (▲) 10, (◆) 20, and (●) 50 g/L initial soluble starch (Ss_0) concentration.

ethanol and the oxidative enzymes were attenuated to restrict glucose oxidation. This was accomplished by not inducing e_I and depressing e_T and e_{II} at low glucose concentrations and inducing e_I and repressing e_T and e_{II} at high glucose concentrations. However, the ethanol produced was oxidized for more recombinant protein, cells and CO_2 synthesis. Inhibition of glucose catabolism by ethanol was insignificant since the ethanol concentration (E) in the liquid medium fell below 40 g/L⁴. Furthermore, the mass balance in Appendix A showed production rate of CO_2 (CPR) was five times greater than that of ethanol ($-r_E$). Hence, most carbon was metabolized to produce CO_2 other than ethanol and biomass (Fig. 9). These findings indicate that the Crabtree effect was not the issue. The possible explanation to the low biomass concentration observed above may have been due to the effect of

dissolved oxygen in the medium, since the mass transfer rate of O_2 from the gas to the liquid (OTR) and the rate of O_2 utilization (OUR) (162 mmol O_2 /L/h) at pseudo-steady state are much larger than the time rate of change in C_L (0.66 mmol O_2 /L/h) (see Appendix A).

The biomass concentration increased with the increase in the initial glucose concentration as shown for the YNB-G and YEP-G media. However, a decline in biomass concentration was observed after 25 h in the YEP-Ss medium. The biomass concentration in YEPG was 2 times greater than that in YEPSs.

The maximum specific growth rates were calculated from the slopes of $\ln(x)$ versus time plots during the exponential phase (Table II). Slightly lower specific growth rates were calculated in the starch-containing medium when compared with the YEP-G medium (Table II). This

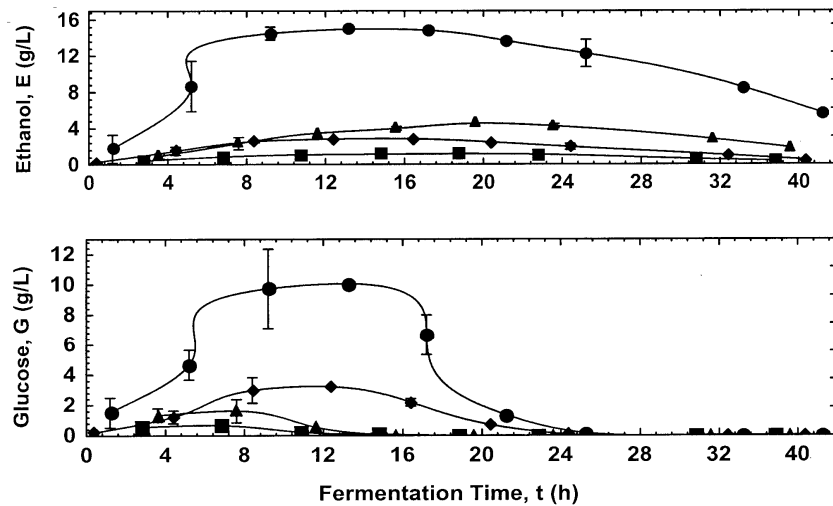


Fig. 7. Fermentation characteristics of *S. cerevisiae* C468/pGAC9 grown in shake flasks in YEP-Ss medium. Time profile of glucose (G) and ethanol (E) concentrations in YEP-Ss medium containing (■) 5, (▲) 10, (◆) 20, and (●) 50 g/L initial soluble starch (S_{s_0}) concentration.

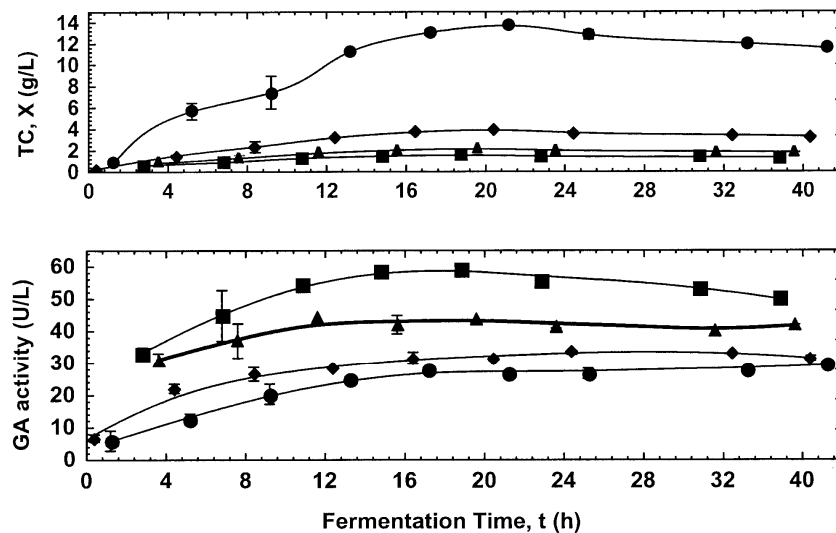


Fig. 8. Fermentation characteristics of *S. cerevisiae* C468/pGAC9 grown in shake flasks in YEP-Ss medium. Time profile of total cell (TC) (X) concentration and glucoamylase (GA) activity in YEP-Ss medium containing (■) 5, (▲) 10, (◆) 20, and (●) 50 g/L initial soluble starch (S_{s_0}) concentration.

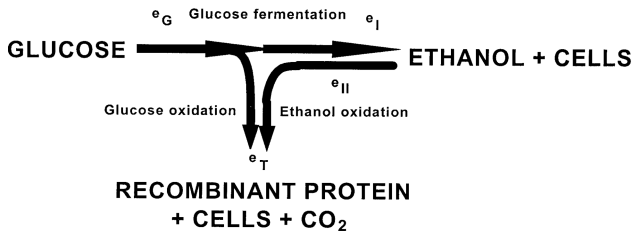
can be explained by the fact that although D-glucose is a readily metabolizable carbon source for *S. cerevisiae*, starch is not, and it has to be hydrolyzed to low molecular weight sugars first and then metabolized. However, the growth rate in YEP-Ss medium containing $S_{s_0} = 50$ g/L was 1.3 times greater than in YEP-G (Table III).

Control experiments carried out by growing the host C468 strain under selective condition (YNB-G selective medium, $G_0 = 20$ g/L) showed no detectable glucoamylase activity and no significant (NS) growth rate when grown on YEP-Ss. For this reason, enzyme activity was used throughout the kinetic studies. The results are summarized in Table II based on the end point of fermentation.

In 40 h flask culture experiments under selective conditions (YNB-G selective medium, $G_0 = 20$ g/L) more than 88% of the population contained plasmids. However, under non-selective condition (YEP-G non-selective medium, $G_0 = 20$ g/L), only over 61% of the population contained the plasmids over all growth phases. This may be attributed to proper partitioning due to large bud sizes associated with low ethanol production observed in these fermentations. In YEP-Ss non-selective medium containing 0.5 and 5% (w/v) starch, 80% of the cells were found to retain their plasmid at the end of the fermentation. These results suggest that the expression of the gene and plasmid stability is interrelated: as the expression of the plasmid gene increases, plasmid stability decreases.

Table II. Shake flask experimental results for YNB-G, YEP-G, and YEP-Ss media.

Medium	Initial glucose or starch (g/L)		μ_{\max} (1/h)	$Y_{X/S}$ (g/g)	$Y_{GA/X}$ (U/g)	$Y_{GA/S}$ (U/g)
YNB-G	5		0.139 ± 0.005	0.545 ± 0.004	25.3 ± 0.491	0.195 ± 0.002
	10		0.174 ± 0.037	0.242 ± 0.006	33.3 ± 0.198	0.411 ± 0.003
	20		0.189 ± 0.002	0.130 ± 0.002	37.2 ± 0.984	0.567 ± 0.071
	50		0.141 ± 0.002	0.122 ± 0.005	73.5 ± 0.446	2.767 ± 0.019
YEP-G	5		0.298 ± 0.001	0.469 ± 0.007	35.2 ± 0.340	0.391 ± 0.003
	10		0.360 ± 0.006	0.222 ± 0.003	31.8 ± 0.172	0.598 ± 0.006
	20		0.377 ± 0.009	0.119 ± 0.001	47.9 ± 0.871	1.252 ± 0.033
	50		0.309 ± 0.008	0.112 ± 0.008	37.8 ± 0.269	0.227 ± 0.016
YEP-Ss	5		0.219 ± 0.006	0.376 ± 0.006	37.2 ± 0.035	16.3 ± 0.013
	10		0.231 ± 0.001	0.244 ± 0.004	65.7 ± 0.062	13.3 ± 0.048
	20		0.243 ± 0.003	0.112 ± 0.001	94.1 ± 0.085	10.2 ± 0.082
	50		0.302 ± 0.008	0.331 ± 0.007	36.3 ± 0.043	10.1 ± 0.073

**Fig. 9.** Simplified representation of the major catabolic pathways in the recombinant *S. cerevisiae* yeast. e_I = alcohol dehydrogenase I (ADH-I), e_{II} = alcohol dehydrogenase II (ADH-II), e_G = glycolytic enzymes, e_T = TCA enzymes.

Therefore, using the ENOL 1 promoter in recombinant protein synthesis has the following advantages. In the early stage of the recombinant *S. cerevisiae* culture, when glucose concentrations in the culture media are maintained at a high level, the expression of plasmid can be repressed. However, the cells can be grown effectively and the plasmid can be maintained quite stably. When the growth of the cells reaches stationary phase, the plasmid gene can be expressed by maintaining glucose concentrations to a lower level. In this manner, the competitive interactions between host and plasmid can be minimized and plasmid stability can be maintained for a long period of time.

Segregational loss rate (S_L) derived from non-selective cultures showed that pGAC9 was more stable in YNB-G. Plasmid-containing cultures of C468/pGAC9 grown on YEP-G showed a significant growth rate advantage over the original host (C468) while the growth rates of C468 and 468/pGAC9 cultures (plasmid-containing) demonstrated that plasmid carriage imposed a significant burden on the host during culture on YNB-G selective medium. Addition of L-histidine to YNB-G cultures did not significantly affect S_L demonstrating that selection because of L-histidine deficiency was not responsible for the improved stability. Plasmid carriage imposed no significant burden in cultures grown on YEP-G medium. The results are summarized in Table IV.

The amount of the glucoamylase secreted into the medium was also determined at regular time intervals. Since *S. cerevisiae* normally secretes only 0.5% of its own proteins to the medium¹⁹, the measurement of extracellular glucoamylase activities was straightforward. Glucoamylase production rate increased substantially during the

exponential phase of growth, then slowed down as the cells entered their early stationary phase of growth, and a slow increase was observed once again when the cells were in their stationary phase. This behavior suggests that glucoamylase production might be characterized as being growth associated.

Whereas higher biomass concentrations were observed at higher glucose concentrations, the expression for the glucoamylase gene was depressed at the early stages of fermentation when the glucose concentration was the highest. The expression of the glucoamylase gene becomes repressed at a later stage of the fermentation when the glucose concentration decreases to about 5 g/L. In media containing 5 g/L glucose and 5 to 50 g/L starch, the expression of the glucoamylase gene was repressed from the early stages, even though the growth levels of the cells were lower. These results show that the expression of the ENOL 1 promoter is regulated by the glucose concentration.

Control experiments with YEPG and YEPSs media in shake flasks displayed stability profiles (Table IV) very similar to those found in YEP-G and YEP-Ss, indicating the negligible effect of increasing concentrations of yeast extract and peptone in these media. The results are summarized in Table IV.

Bioreactor batch experiments

Since plasmid stability and protein production from different media are the primary concerns, recombinant yeast cells were further investigated in a standard stirred tank bioreactor system. The choice of medium composition was based on the results of shake flask experiments. Since yeast minimal medium (YNB-G) was found to be limited by other nutrients at high D-glucose concentrations, YEPG was chosen for further investigation. Moreover, since experiments in complex media with 5 to 50 g/L initial starch concentration (YEP-Ss) exhibited high plasmid instabilities in shake flask experiments, lower starch concentrations (5 to 30 g/L) were used in YEPSs media. The yeast strain was grown in a stirred tank bioreactor operating in batch mode at 300 rpm and 30°C. The experimental results are summarized in Table V based on the end point of fermentation obtained. Similar growth profiles were observed in bioreactor and shake flask experiments. The highest maximum specific growth rate [$\mu_{\max} = 0.502$ to 0.006 (1/h)] was calculated in YEPG medium containing 5% D-glucose. More than 60% of the

Table III. Shake flask experimental results for YEPG and YEPSs media.

Medium	Initial glucose or starch (g/L)	X (g/L)	μ_{\max} (1/h)	E (g/L)	GA (U/L)	F ⁺ (%)
YEPG	5	3.6 ± 0.053	0.264 ± 0.002	1.1 ± 0.072	46.8 ± 0.043	75 ± 1
	10	5.0 ± 0.044	0.418 ± 0.003	3.2 ± 0.007	96.4 ± 0.072	84 ± 5
	20	7.3 ± 0.016	0.319 ± 0.002	4.4 ± 0.011	193.2 ± 0.071	87 ± 3
	50	15.1 ± 0.012	0.251 ± 0.016	22.7 ± 0.019	167.0 ± 0.154	96 ± 2
YEPSs	5	1.7 ± 0.046	0.233 ± 0.006	1.3 ± 0.039	63.4 ± 0.064	95 ± 6
	10	2.0 ± 0.016	0.246 ± 0.003	3.1 ± 0.072	142.7 ± 0.066	86 ± 3
	20	2.3 ± 0.033	0.270 ± 0.001	4.9 ± 0.056	222.1 ± 0.049	77 ± 1
	50	14.6 ± 0.081	0.322 ± 0.006	16.0 ± 0.022	497.4 ± 0.041	91 ± 4

Table IV. Growth rates and plasmid retention in flask culture experiments.

Medium	C468		C468/pGac9			
	μ_{\max} (1/h)	GA (U/L)	μ_{\max}^+ (1/h)	$\langle \mu_{\max} \rangle$ (1/h)	Segregational loss rate (S_L) (1/generation)	GA (U/L)
YNB-G	0.293 ± 0.004	-	0.176 ± 0.002	0.163 ± 0.004	0.094 ± 0.002	93.5 ± 0.026
YEP-G	0.354 ± 0.003	113.5 ± 0.025	0.308 ± 0.007	0.239 ± 0.001	0.183 ± 0.002	180.1 ± 0.074
YEP-Ss	NS	ND	0.133 ± 0.006	0.081 ± 0.005	0.125 ± 0.009	125.2 ± 0.029

NS = not significant.
 ND = not determined.

Table V. Bioreactor experimental results for YEPG and YEPSs media.

Medium	Initial glucose or starch (g/L)	μ_{\max} (1/h)	$Y_{X/S}$ (g/g)	$Y_{GA/X}$ (U/g)	$Y_{GA/S}$ (U/g)	F ⁺ (%)
YEPG	50	0.329 ± 0.006	0.103 ± 0.079	99.33 ± 0.154	0.979 ± 0.003	90 ± 4
YEPSs	5	0.038 ± 0.008	0.340 ± 0.046	38.62 ± 0.139	14.5 ± 0.074	86 ± 2
	20	0.034 ± 0.008	0.100 ± 0.011	22.84 ± 0.756	2.5 ± 0.049	54 ± 1
	30	0.036 ± 0.007	0.131 ± 0.017	9.18 ± 0.956	1.1 ± 0.085	63 ± 1

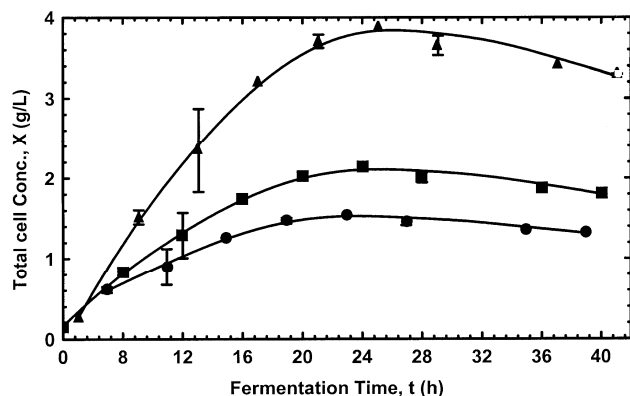


Fig. 10. Fermentation characteristics of *S. cerevisiae* C468/pGac9 grown in a 2-L bioreactor in starch (YEPSs) medium. Time profile of biomass concentration in YEPSs medium containing (▲) 30, (■) 20, and (●) 5 g/L initial starch (S_{s0}) concentration. Total cell concentration, $X = X^+ + X^-$.

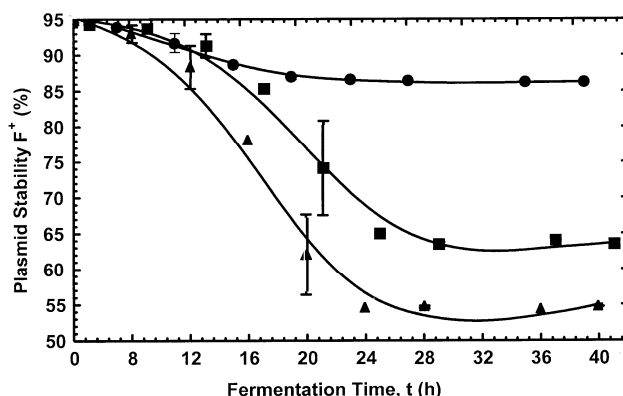


Fig. 11. Fermentation characteristics of *S. cerevisiae* C468/pGac9 grown in a 2-L bioreactor in starch (YEPSs) medium. Time profile of plasmid stability in YEPSs medium containing (▲) 30, (■) 20, and (●) 5 g/L initial starch (S_{s0}) concentration.

cells retained their plasmids at the end of ca. 40 h and 99.3 units of glucoamylase were secreted per g cell mass. The use of low concentrations of starch (5 to 30 g/L) in the YEPSs medium enhanced biomass yield relative to shake flask experiments. Surprisingly, when the starch concentration was increased from 5 g/L to 20 g/L, or 30 g/L in bioreactor experiments, a drastic loss was observed in plasmid stability (Table V). Comparing the stabilities obtained in different concentrations of starch revealed a negative correlation between them. Only 63% of cells were found to contain their plasmids at the end of ca. 40 h of fermentation time in YEPSs containing 30 g/L starch.

Figs. 10–12 show the time course of biomass, plasmid stability and glucoamylase production of the C468/pGac9 strain grown in YEPSs medium.

The biomass concentration followed its regular S shaped pattern over the fermentation period except for slight increases when the cells were grown in YEPSs with 5 g/L and 30 g/L of initial starch. There was no decline in the biomass concentration of the culture in the late exponential and stationary phases of growth. It is also interesting to note that extracellular glucoamylase activity, which is directly affected by plasmid loss, still remained at a reasonable level. The ratio of plasmid-free cells to the

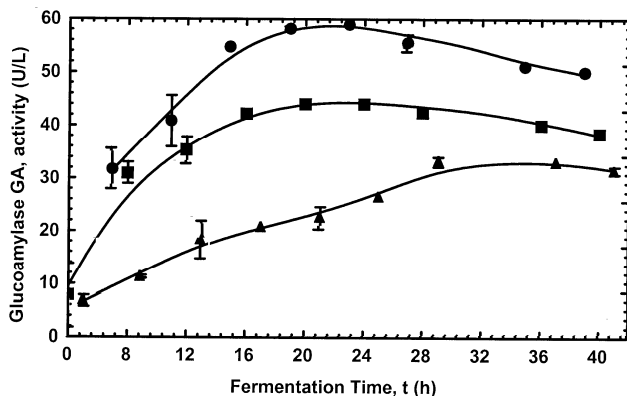


Fig. 12. Fermentation characteristics of *S. cerevisiae* C468/pGAC9 grown in a 2-L bioreactor in starch (YEPSs) medium. Time profile of glucoamylase (GA) activity in YEPSs medium containing (\blacktriangle) 30, (\blacksquare) 20, and (\bullet) 5 g/L initial starch (S_{s0}) concentration.

plasmid-bearing cells increased during the exponential and late exponential phases of the batch growth in both bioreactor and shake flask experiments⁷. The cells had a reduced growth rate when grown in YEPSs media with different starch concentrations. The amount of secreted glucoamylase also decreased with increasing starch concentration in the medium. High plasmid stabilities observed in non-starch media were associated with high growth rates. Higher growth rates in starch gave lower plasmid stability. In contrast, higher stabilities in non-starch media were associated with high growth rates. It has been postulated that plasmid stability would increase with higher growth rates since proper partitioning would be more likely due to larger bud sizes¹⁰. Plasmid instability observed in starch-containing media may be due to the promoter, ENOL 1, which is a strong yeast promoter inducible by the substrate and strongly affected by the presence of low levels of glucose¹³ in the medium. Higher plasmid stability (Table V) was observed in YEPSs medium containing 50 g/L initial starch concentration as compared to low initial starch concentrations (5 to 30 g/L). This was attributed to high growth rates associated with increased glucose concentrations in such media.

Our findings have suggested that the low conversion efficiency of starch to ethanol by the recombinant yeast C468/pGAC9 strain may be due to plasmid instability. At low plasmid stability, the conversion of starch into reducing sugars becomes unfavorable due to the low levels of secreted glucoamylase activity, leading to low ethanol concentrations. It was previously confirmed¹⁴ that the C468/pGAC9 strain is capable of producing ethanol from starch if appropriate conditions are provided; the present study leads to a better understanding of the effects of carbon source and its concentration on the plasmid stability and growth of the recombinant yeast strain. The optimization of both plasmid stability and ethanol production with high yield from starch biomass appears to be a challenge and further studies are encouraged.

Kinetic model study

The commonly proposed kinetic models for enzyme production are listed in Table VI. The first two models in

Table VI. Kinetic models applied to microbial glucoamylase production.

Model	Formula	Reference
Brown-Vass	$\frac{dGA}{dt} = k_p \frac{dX}{dt} t^{-t_m}$	Brown and Vass ¹⁵
Kono-Asai	$\frac{dGA}{dt} = k_p \frac{dX}{dt}$	Kono and Asai ¹⁴
Modified Montesinos	$\frac{dGA}{dt} = k_m \frac{GA}{K_s + GA} X$	Montesinos et al. ¹³

Table VI are linear models, and therefore, their parameters were estimated using linear least squares methods. The last model is a non-linear one, and the steepest descent method was used to estimate its parameters. Although it is possible to convert the non-linear least squares problem for the last model to a linear one by considering the reciprocal of dGA/dt , it was found to be numerically unstable, yielding a higher σ^2 than the steepest descent method for the original variables. The cost function used in the optimization of parameters was the sum of the squares of the differences in time derivatives of the experimental data and their estimated derivatives of enzyme concentration. In the modified Montesinos model¹² due to the nature of the steepest descent method, the converged results depend on the initial guess for the parameters and the step size for iterations. Parameter values were therefore checked for robustness due to neighboring starting guesses and different step sizes. The number of data points also affected the residual sum of the squares of the errors of a fit.

In the Kono-Asai model, only the exponential phase of growth was considered for parameter estimation. In this model the interactions of product formation and biomass growth rates were assumed to occur simultaneously. On the other hand, in the Brown-Vass model the rate of glucoamylase production was related to the delayed rate of biomass formation, termed as the maturation time (t_m) by definition, a parameter which has to be optimized first. The Brown-Vass model was applicable throughout all the growth regions. The Montesinos model¹², originally proposed for modeling lipase production by *Candida rugosa*, was simplified by neglecting the effect of the incremental change in substrate concentration.

Results obtained from shake flask experiments for the growth of C468/pGAC9 in YNB-G, YEP-G and YEP-Ss media were utilized in examining various kinetic models. The choice of the experimental data was based on the high plasmid stabilities displayed in each medium type, since the unstructured kinetic models utilized did not consider any plasmid instability. Model parameters are tabulated in Table VII, and glucoamylase concentration versus time plots are given in Figs. 13–15 for the growth of recombinant yeast cells in YNB-G and YEP-G containing 20 g/L initial D-glucose and YEP-Ss media containing 20 g/L initial starch. As a rule of thumb, if one encounters a σ^2 value above 5%, the estimated model parameter(s) is (are) said to be acceptable in order to test the model.

The correlation coefficients for the following cases were above 90%: Kono-Asai model in 20 g/L initial

Table VII. Kinetic parameters for C468/pGAC9 grown on YNB-G selective medium containing 20 g/L D-glucose, YEP-G non-selective medium containing 20 g/L D-glucose, and YEP-Ss non-selective medium containing 20 g/L starch initially.

Model	Parameter	Parameter value		
		YEP-G	YEP-Ss	YNB-G
Brown-Vass	k_p (U/g)	23.1 ± 0.13	31.4 ± 0.52	42.0 ± 0.22
	t_m (h)	8.5 ± 0.44	8.1 ± 0.91	7.9 ± 0.11
Kono-Asai	k_p (U/g)	0.063 ± 0.03	0.153 ± 0.0117	0.045 ± 0.015
Modified Montesinos	k_{max} (U/g)	0.8 ± 0.33	0.6 ± 0.45	1.3 ± 0.22
	K_s (U/L)	6.7 ± 0.63	12.6 ± 0.34	25.1 ± 0.58

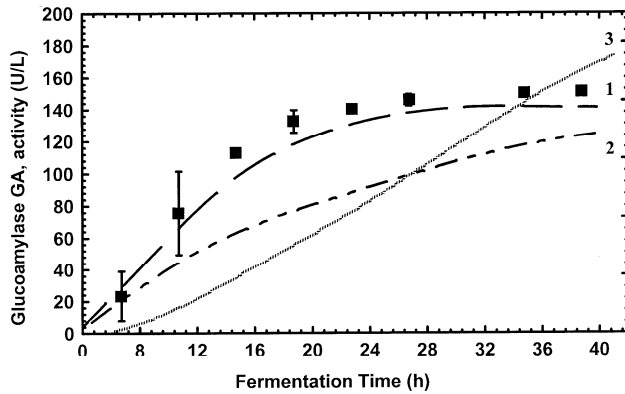


Fig. 13. Model predictions of glucoamylase activities for *S. cerevisiae* C468/pGAC9 in YNB-G medium containing (G_0) 20 g/L initial D-glucose. (⊗) Experimental; Model: (1) Brown-Vass, (2) Kono-Asai, (3) Montesinos.

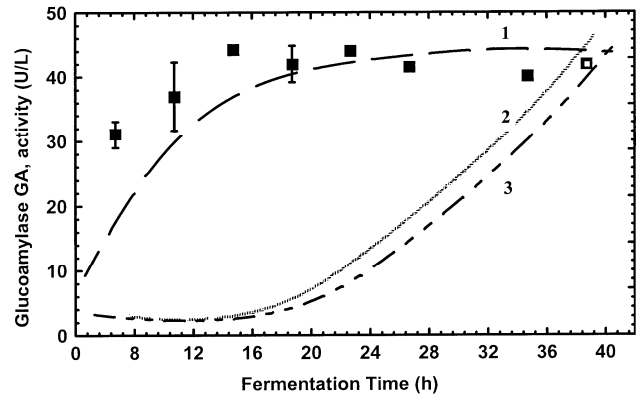


Fig. 15. Model predictions of glucoamylase activities for *S. cerevisiae* C468/pGAC9 in YEP-Ss medium containing (S_0) 20 g/L initial soluble starch. (⊗) Experimental; Model: (1) Brown-Vass, (2) Kono-Asai, (3) Montesinos.

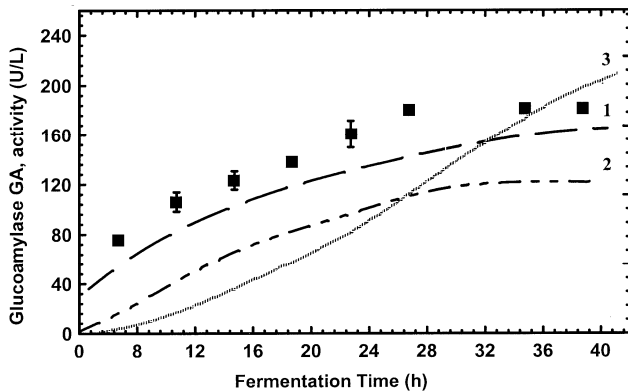


Fig. 14. Model predictions of glucoamylase activities for *S. cerevisiae* C468/pGAC9 in YEP-G medium containing (G_0) 20 g/L initial D-glucose. (⊗) Experimental; Model: (1) Brown-Vass, (2) Kono-Asai, (3) Montesinos.

glucose concentration; Brown-Vass model in 10 g/L, 20 g/L and 50 g/L initial glucose concentrations; and the modified Montesinos model in all initial D-glucose concentrations. The correlation coefficient for the Brown-Vass model in 50 g/L initial D-glucose concentration and for the Montesinos model in 10 g/L and 20 g/L initial D-glucose concentrations were particularly high (above 99%).

Among the models tested for YNB-G and YEP-G media, Kono-Asai and the modified Montesinos showed poor fits although they had high correlation coefficients. The correlation coefficient of the former model accounts only for linear relationships, while that of the latter model accounts for non-linear relationships. The Brown-Vass

model on the other hand gave a good fit starting from the beginning of the exponential phase until the end of the stationary phase. The model of the modified Montesinos described glucoamylase production starting from the lag phase until the end of exponential phase, and then showed an overshoot. This may be due to the consideration of only a specified growth region. Furthermore, the number of data points after the exponential phase was only three, which may also contribute to the less accurate fits at the stationary phase. The model of Kono-Asai underestimated the experimental data. The experimental observation that glucoamylase production is likely to be growth associated was also supported by the modified Montesinos model. All the models were able to describe glucoamylase production over the entire fermentation process, but were less accurate in simulating the glucoamylase activity, in starch containing medium (YEPSs), except the Brown-Vass model which covered a wide range of data, starting from the lag phase until the end of stationary phase.

Comparing the fits, the kinetic models of Brown-Vass and the modified Montesinos, the first one being linear and the second one being non-linear, were best among the models studied for describing the experimental data on extracellular glucoamylase activity in different media^{2,12}. The results represented in this study may find applications in modeling starch fermentation by recombinant yeast cells including possible effects associated with plasmid stability.

CONCLUSIONS

The recombinant *S. cerevisiae* strain (C468/pGAC9) secreting a protein displaying glucoamylase activities was

investigated in terms of its biomass growth, glucoamylase production and plasmid stability in different compositions of media containing either glucose or starch initially. The carbon source and its concentration in the medium affected the plasmid stability of the strain. Plasmid stability was substantially increased at high growth rates, though not with starch. Almost no plasmid loss was observed when minimal media (YNB-G) with different initial D-glucose concentrations, and two complex media containing glucose (YEP-G and YEPPG), were used to grow the recombinant strain, while a considerable level of plasmid instability was recorded when it was grown in complex media containing starch (YEP-Ss) in both shake flasks and in a batch bioreactor. The results show that the plasmid pGAC9 was subject to segregational instability even in batch cultures contrary to the expectation of insignificant segregation instability in such cultures. The kinetics of glucoamylase synthesis were found to be cell growth associated. The unstructured kinetic model tested gave a good prediction for glucoamylase production associated with biomass formation. The kinetic analysis presented here may provide a basis for future modeling of the recombinant system and may find application in the mathematical modeling of starch fermentation by recombinant yeast cells.

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NOMENCLATURE

a	interfacial mass transfer area (m^2/m^3)
b_2	constant (g dcw)
CFU	colony forming units
C_L	O_2 concentration in liquid medium (mmol/L)
C_L^*	O_2 saturation in liquid medium (mmol/L)
C_0^*	O_2 saturation in liquid medium at start of fermentation (mmol/L)
CPR	CO_2 production rate (mmol $CO_2/g/h$)
D	dianisidine
DO_2	dissolved oxygen (-)
d	light path length (cm)
E	ethanol concentration (g/L)
e_G	glycolytic enzyme pool specific concentration relative to level at balanced growth (-)
e_I	ADH-I enzyme pool specific concentration relative to level at full induction and balanced growth (-)
e_{II}	ADH-II enzyme pool specific concentration relative to level at full depression and balanced growth (-)
e_T	TCA enzyme pool specific concentration relative to level at full depression and balanced growth (-)
G	D-glucose concentration (g/L)

G_0	initial D-glucose concentration (g/L)
GOD	glucose oxidase enzyme
F^+	fraction of plasmid-carrying cells (%)
GA	glucoamylase concentration (U/L)
I	iodine
k_1	rate constant for glucose fermentation (h^{-1})
k_2	rate constant for glucose oxidation (h^{-1})
k_3	rate constant for ethanol oxidation (h^{-1})
K_1	Michaelis constant for glucose fermentation (g/L)
K_2	Michaelis constant for glucose oxidation (g/L)
K_3	Michaelis constant for ethanol oxidation (g/L)
KI	potassium iodide
k_L	oxygen mass transfer coefficient in liquid medium (cm/h)
k_{max}	maximum rate coefficient (U/g h)
k_p	product formation rate constant (U/g h)
K_s	saturation constant (U/g)
M	maltose concentration (g/L)
NAD	nicotinamide dinucleotide
NADH	nicotinamide dinucleotide hydrogen (reduced-NAD)
OD	optical density
P	peptone concentration (g/L)
PPi	phospho-pyrophosphate
POD	pero-oxidase enzyme
S	substrate concentration (g/L)
S_L	segregational loss rate (1/generation)
Ss	soluble starch concentration (g/L)
Ss_0	initial soluble starch concentration (g/L)
R	regression coefficient
$-r_E$	rate of ethanol consumption (g/L)
$-r_G$	rate of glucose consumption (g/L)
r_1	rate of dcw synthesis from glucose fermentation (g dcw/L/h)
r_2	rate of dcw synthesis from glucose oxidation (g dcw/L/h)
r_3	rate of dcw synthesis from ethanol oxidation (g dcw/L/h)
r_4	rate of anabolism (g dcw/L/h)
t	time (h)
t_m	maturation time (h)
X	total cell concentration (g/L)
X_c	dry cell mass weight fraction of carbon (g carbon/g dcw)
Y_{ESF}	yield of ethanol from fermented glucose (g ethanol/g glucose)
Y_{XE}	yield of cell mass from ethanol (g dcw/g ethanol)
Y_{XSF}	yield of cell mass from fermented glucose (g dcw/g glucose)
Y_{XSO}	yield of dry cell mass from oxidized glucose (g dcw/g glucose)
X^+	plasmid-carrying cell concentration (g/L)
X^-	plasmid-free cell concentration (g/L)
YE	yeast extract concentration (g/L)
YNB	yeast nitrogen base concentration (g/L)
$Y_{X/S}$	yield coefficient for total cells (g/g)

$Y_{GA/S}$ yield coefficient for glucoamylase (U/g)
 $Y_{GA/X}$ specific yield coefficient for glucoamylase (U/g)

GREEK

$\Sigma CC_{selective}$ summation of the counts of colonies on selective medium
 $\Sigma CC_{non-selective}$ summation of the counts of colonies on non-selective medium
 ε absorbance coefficient (cm²/μmole)
 μ_{max} maximum specific growth rate (1/h)
 σ^2 variance

REFERENCES

- Bannister, S.J. and Wittrup, K.D., Glutathione expression in response to heterologous protein secretion in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.*, 2000, **68**(4), 879-809.
- Brown, D.E. and Vass, R.C., Maturity and product formation in culture of microorganisms. *Biotechnol. Bioeng.* 1973, **15**, 321-330.
- Cole, G.E., McCabe, P.C., Inlow, D., Gelfand, D.H., Ben-Bassat, A. and Innis, M.A., Stable expression of *Aspergillus awamori* glucoamylase in distiller's yeast. *Bio/Technol.*, 1988, **6**, 417-421.
- Coppella, S.J. and Dhurjati, P.A, A mathematical description of recombinant yeast. *Biotechnol. Bioeng.*, 1990, **35**, 356-374.
- De Moraes, L.P.M., Astoltofi-lho, S. and Oliver, S.G., Development of yeast strains for the efficient utilization of starch: evaluation of constructs that express α -amylase and glucoamylase separately or as bifunctional fusion proteins. *Appl. Microb. Biotechnol.*, 1995, **143**(6), 1067-1076.
- Gorgens, J.F., van Zyl, W.H. and Knoetze, J.H., The metabolic burden of the *PGK1* and *ADH2* promotor system for heterologous xylanase production by *Saccharomyces cerevisiae* in defined medium. *Biotechnol. Bioeng.*, 2001, **73**(5), 238-245.
- Gupter, J.C., Pandey, G. and Mukherjee, K.J. , Two-stage cultivation of recombinant *Saccharomyces cerevisiae* to enhance plasmid stability under non-selective conditions: Experimental study and modeling. *Enzyme Microbiol. Technol.*, 2001a, **28**, 89-99.
- Hardjito, L., Greenfield, P.F. and Lee, P.L., A model for β -galactosidase production with recombinant yeast *Saccharomyces cerevisiae*. *Enzyme Microbiol. Technol.*, 1993, **15**, 120-126.
- Innis, M.A., Holland, M.J., McCabe, P.C., Cole, G.E., Wittman, V.P., Tai, R., Watt, K.W.K., Gel, F.D.H., Holland, J.P. and Meade, J.H., Expression, glycosylation, and secretion of an *Aspergillus* glucoamylase by *Saccharomyces cerevisiae*. *Science*, 1985, **228**, 21-26.
- Kilonzo, P., Margaritis, A., Yu, J.T. and Ye, Q., Bioethanol production from starchy biomass by direct fermentation using *Saccharomyces diastaticus* in batch free and immobilized cell systems. *Intern. J. Green Energy*, 2007, **4**, 1-14.
- Kono, T. and Asai, T., Kinetics of fermentation processes. *Biotechnol. Bioeng.*, 1969, **11**, 293-321.
- Montesinos, J.L., Lafuente, J., Gordilla, M.A., Valero, F., Sola, C., Charbonnier, S. and Cheruy, A., Structured modeling and state estimation in a fermentation process: lipase production by *Candida rugosa*. *Biotechnol. Bioeng.*, 1995, **48** 573-584.
- Nunberg, J., Flatgaard, J.E. and Innis, M.A., Glucoamylase c-DNA. U.S. Patent 4,794,175, Cetus Corporation, Emeryville, CA, 1988.
- O'Kennedy, R.D. and Patching, J.W., Effects of medium composition and nutrient limitation on loss of the recombinant plasmid pLG669-z and β -galactosidase expression by *Saccharomyces cerevisiae*. *J. Ind. Microb. Biotechnol.*, 1997, **18**, 319-325.

- O'Kennedy, R.D., Houghton, C.J. and Patching, J.W., Effects of growth environment on recombinant plasmid stability of *Saccharomyces cerevisiae* grown in continuous culture. *Appl. Microbiol. Biotechnol.*, 1995, **44**, 126-132.
- Wang, Z. and DaSilva, N.A., Improved protein synthesis and secretion through medium enrichment in a stable recombinant yeast strain. *Biotechnol. Bioeng.*, 1993, **42**, 95-102.
- Zanone, J.P., Peres, M.F.S. and Gattas, E.A.L., Colorimetric assay of ethanol using alcohol dehydrogenase from dry baker's yeast. *Enzyme Microb. Technol.*, 2007, **40**, 466-470.
- Zhang, X. Z. Xia, Zhao, B. and Cen, P., Enhancement of plasmid stability and protein productivity using multi-pulse, fed-batch culture of recombinant *Saccharomyces cerevisiae*. *Biotechnol. Lett.*, 2002, **24**, 995-998.
- Zhang, Z., Scharer, J.M. and Moo-Young, M., Plasmid instability kinetics in continuous culture of a recombinant *Saccharomyces cerevisiae* in airlift bioreactor. *J. Biotechnol.*, 1997a, **55**, 31-41.
- Zhang, Z., Scharer, J.M. and Moo-Young, M., Mathematical model for aerobic culture of a recombinant yeast. *Bioprocess Eng.*, 1997, **17**, 235-240.

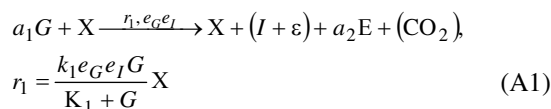
(Manuscript accepted for publication May 2008)

APPENDIX A

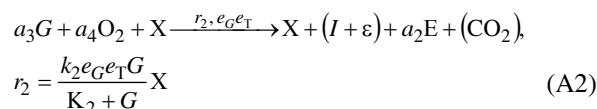
Mass balance

The stoichiometry and rate expression for the three catabolisms represented in Fig. 9 were:

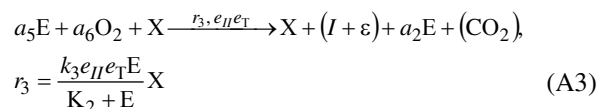
Glucose fermentation:



Glucose oxidation:



Ethanol oxidation:



where I is the concentration of metabolic intermediates and ε is biochemical energy. The rates of glucose and ethanol utilization are

$$-\frac{dG}{dt} = (-r_G) = a_1 r_1 + a_3 r_2 \quad (A4)$$

$$-\frac{dE}{dt} = (-r_E) = a_5 r_3 - a_2 r_1 \quad (A5)$$

$$\frac{dC}{dt} = (r_O) = \frac{k_L a}{V} (C_L^* - C_L) - OUR \quad (A6)$$

A pseudo-steady state approximation of C_L and C_L^* is

$$C_L = C_L^* - \frac{\text{OUR}V}{k_L a} \quad (\text{A7})$$

$$C_L^* = C_0^* \left(1 - \frac{X}{b_2} \right) \quad (\text{A8})$$

The DO_2 is thus

$$\text{DO}_2 = \frac{C_L^*}{C_0^*} - \frac{\text{OUR}V}{C_0^* k_L a} \quad (\text{A9})$$

Neglecting changes in the dissolved oxygen, from stoichiometry the oxygen uptake rate (OUR) was

$$\text{OUR} = 1000(a_4 r_2 + a_6 r_3) \quad (\text{A6})$$

A carbon balance yields the following for the carbon dioxide (CO_2) production rate (CPR):

$$\text{CPP} = \frac{1000[0.3978(-r_G) + 0.5217(-r_E) - X_c r_4]}{12} \quad (\text{A7})$$

where

$$a_1 = \frac{1}{Y_{\text{XGF}}} \quad (\text{A8})$$

$$a_2 = a_1 Y_{\text{EGF}} \quad (\text{A9})$$

$$a_3 = \frac{1}{Y_{\text{XGO}}} \quad (\text{A10})$$

$$a_4 = a_3 \cdot 1 \text{ g O}_2 / \text{g glucose} - 0.33 \text{ g O}_2 / \text{g dcw} \quad (\text{A11})$$

$$a_5 = \frac{1}{Y_{\text{XE}}} \quad (\text{A12})$$

$$a_6 = a_5 \cdot 2.09 \text{ g O}_2 / \text{g ethanol} - 0.33 \text{ g O}_2 / \text{g dcw} \quad (\text{A13})$$

$$r_1 = \frac{k_1 E_G E_I G_1 X}{K_1 + G_1} \quad (\text{A14})$$

$$r_2 = \frac{k_2 E_G E_T G_2 X}{K_2 + G_2} \quad (\text{A15})$$

$$r_3 = \frac{k_3 E_{II} E_T E}{K_3 + E} \quad (\text{A16})$$

Therefore,

$$\begin{aligned} (-r_G) &= a_1 r_1 + a_3 r_2 = 6.5 * 0.38 + 2.0 * 0.25 \\ &= 3.88 (\text{g glucose} / \text{L} / \text{h}) \end{aligned}$$

$$\begin{aligned} (-r_E) &= a_5 r_3 - a_2 r_1 = 1.9 * 0.06 - 3.0 * 0.38 \\ &= 1.54 (\text{g EtOH} / \text{L} / \text{h}) \end{aligned}$$

$$\begin{aligned} \text{OUR} &= 1000(a_4 r_2 + a_6 r_3) = 1.67 * 0.25 + 79.74 * 0.06 \\ &= 5202 \text{ mmol O}_2 / \text{L} / \text{h} (166.5 \text{ g O}_2 / \text{L} / \text{g}) \end{aligned}$$

$$\begin{aligned} \text{CPR} &= 1000[0.3978 * (2.97) + 0.5217(1.254) - 0.44 * 0.02] / 12 \\ &= 53.1 \end{aligned}$$

Appendix Table A. Summary of parameters used.

Parameter		
a_1	8.93	(g glucose/g dcw) (This work)
a_2	2.44	(g glucose/g dcw) (This work)
a_3	2.00	(g glucose/g dcw) ¹⁹
a_4	1.67	(g O ₂ /g dcw) ¹⁹
a_5	38.31	(g ethanol/g dcw)(This work)
a_6	79.74	(g O ₂ /g dcw)(This work)
b_2	35	(g dcw) ¹⁹
e_I	1.0	(dimensionless) ¹⁹
e_{II}	1.0	(dimensionless) ¹⁹
e_G	1.0	(dimensionless) ¹⁹
e_T	1.0	(dimensionless) ¹⁹
E	0.018	(g/L) (This work)
G_1	2.2	(g/L) (This work)
G_2	0.0074	(g/L) (This work)
k_1	0.44	(g/L) ¹⁹
k_2	0.25	(g/L) ¹⁹
k_3	0.10	(g/L) ¹⁹
K_1	2.1	(g/L) ¹⁹
K_2	0.005	(g/L) ¹⁹
K_3	0.48	(g/L) ¹⁹
$k_{L,a}$	98	(1/h) ¹⁹
r_1	0.38	(g dcw/L/h) (This work)
r_2	0.25	(g dcw/L/h) (This work)
r_3	0.06	(g dcw/L/h) (This work)
r_4	0.02	(g glucose/L/h) ¹⁹
$-r_E$	1.54	(g/L) (This work)
$-r_G$	3.88	(g/L) (This work)
V	0.3, 1.2	L (This work)
x_c	0.44	(g/L) ¹⁹
X	15.1	(g/L) (This work)
Y_{EGF}	0.274	(g E/g glucose) (This work)
Y_{XGF}	0.112	(g dcw/g glucose) (This work)
Y_{XE}	0.026	(g dcw/g ethanol) (This work)
Y_{XGO}	0.5	(g E/g oxidized glucose) ¹⁹