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Recombinant sweet potato sporamin production via glucose/pH control in fed-batch cultures of *Saccharomyces cerevisiae*

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Abstract

Sporamin is a storage protein in sweet potato tubers. In batch fermentation of recombinant *Saccharomyces cerevisiae* using selective yeast nitrogen base, the specific trypsin inhibitory activity of sporamin can reach a level of 463 U/g-cell and total volumetric activity of 1574 U/l. However, using non-selective YPAD medium (20 g/l glucose, 10 g/l yeast extract, 10 g/l peptone and 10 g/l ammonia sulphate), only lower specific activity of 344 U/g-cell but higher total activity of 2923 U/l was obtained. Using YPAD medium in fed-batch fermentation and controlling for different glucose concentrations within a specific pH range to reduce the accumulation of ethanol, glycerol and acetate, a specific sporamin activity of 347 U/g-cell and a total activity 6212 U/l was obtained with a glucose concentration of 1 g/l. An increase of specific activity to 831 U/g-cell and a total activity 12 222 U/l was obtained with a glucose concentration of 0.1 g/l.

Keywords: Recombinant sporamin; Fed-batch culture; Glucose/pH control; *Saccharomyces cerevisiae*; Trypsin inhibitors; Non-selective medium

1. Introduction

Sporamin is a soluble protein in sweet potatoes, which accounts for 0.3–2% on a fresh root basis, depending on the strain and/or seasonal variation [1,2]. The predicted protein sequence of sporamin has a common amino acid sequence identity with some Kunitz-type trypsin inhibitors [3]. In addition, sporamin demonstrates a direct defensive role in plants against insect infestations, and insect-resistant transgenic cauliflower plants expressing the sporamin gene have been developed [5]. There have also been many attempts to exploit the cancer therapies of protease inhibitors based on inhibiting the growth of tumour or transformed cells [2,4].

A full length of 0.54 kb cDNA encoding sporamin from sweet potato has previously been ligated to glutathione S-transferase (GST) and transformed into *Escherichia coli* XL1 Blue to produce the fusion protein of GST-sporamin [3]. The authors also developed control strategies for the fed-batch fermentation to enhance the productivity of the GST-sporamin fusion protein [6]. *E. coli* is an effective host for the mass production of recombinant protein, but for future applications of recombinant sporamin in agricultural, medical or health-care markets, and also for eukaryote-specific post-modification, *Saccharomyces cerevisiae* would be a preferred host [7,8].

There has been only one report showing sporamin-related polypeptides expressed in the *pep4* mutant *S. cerevisiae*, but no data was presented to show the trypsin inhibitory activity of those polypeptides [9]. There have also been studies concerning the major factors, i.e. genetics, microbial physiology, fermentation process design and immobilised cell system, that affect the productivity of heterologous protein by *S. cerevisiae* [7,10]. When yeast mutants of amino acid auxotrophy are used in continuous or fed-batch culture in large-scale production, it is important to use a non-selective medium, while also maintaining plasmid stability and high-level expression. Dissolved oxygen tension and dilution rate have been manipulated to increase recombinant protein production in a non-selective medium

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[11–13]. A two-stage cultivation strategy was designed as a selective medium for use in the initial batch stage, followed by periodic glucose starvation [14] or yeast extract (leucine) starvation feeding strategy [15]. The two-stage process usually resulted in a low cell growth condition. Various feeding methods during recombinant yeast fermentation were used to control the concentration of carbon source based on respiratory quotient [16], dissolved oxygen change [17,18,21], dilution rate [19], glucose feed rate (open loop control) [20], substrate consumption modelling [22,23], and off-line analysis [24]. Most studies mentioned that the carbon source and the ethanol concentration produced were critical for maximum production of heterologous protein. In this study, an expression cassette containing glyceraldehydes-3-phosphate dehydrogenase (GAPDH) promoter and *trp1* selection marker have been designed and constructed to overproduce recombinant sporamin in *S. cerevisiae* KCl. A feeding strategy based on the close-loop glucose and pH control to reduce the accumulation of ethanol, glycerol and acetate in fed-batch recombinant yeast culture was developed, achieving high sporamin productivity by using low-cost rich (non-selective) media in a one-stage process.

2. Materials and methods

2.1. Strains and plasmids

S. cerevisiae KCl (*Mat* α, *trp1*, *pep4*) strain was used as host. The recombinant plasmid pYE8 consists of the constitutive GAPDH promoter, the gene encoding sporamin, (cDNA–trypsin inhibitor A (TIA; pSPTi-1 cDNA, accession No. U17333) from *Ipomoea batatas* Lam), 2 μm yeast replicating origin, *trp1* gene as selective marker, autonomously replicating sequences (ARS) and ampicillin resistance gene.

2.2. Medium and cultivation

YNBD medium (1.7 g/l yeast nitrogen base without amino acid and ammonium sulphate, 5 g/l ammonium sulphate and 20 g/l glucose) was used for selection of plasmid-carrying yeast and for the seed culture. A single colony of recombinant yeast on YNBD agar plate was inoculated into 10 ml YNBD medium and incubated overnight at 30 °C. The seed was transferred into a 500 ml Erlenmeyer flask containing 250 ml YNBD medium and incubated at 30 °C and a shaking rate of 180 rpm until an OD_{600} of 1.0 was reached. This culture was used as a seed for batch or fed-batch fermentation. Two types of non-selective medium, i.e. YPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) and YPAD medium (10 g/l yeast extract, 10 g/l peptone, 10 g/l ammonium sulphate and 20 g/l glucose) were used in shake flask, batch or fed-batch culture. The variation of yeast extract (YE) concentration (0.5, 1 or 1.5% YE) in YPD medium was also used in shake flask experiments.

2.3. Batch and fed-batch fermentation

The seed culture of 250 ml was transferred to a 6 l jar fermentor (Firstek, Taiwan) containing 2.25 l of selective or non-selective medium. The culture conditions for batch fermentation were: agitation, 500 rpm; air flow rate, 10 l/min; temperature, 30 °C; initial pH, 5.5 as controlled within 5.5 ± 0.5 by adding 5 N NaOH or 5 N HCl solution; initial dissolved oxygen (DO), 100% saturation referred to air. The fed-batch culture had the same initial operational conditions as in batch culture. It then followed an on-line data acquisition and control strategy written in an icon-based graphical program (GENIE, Advantech, Taiwan) for glucose, pH and DO control. Fermentation broth was continuously circulated outside the fermentor to a microfilter and the filtrate was pumped to the glucose analyser (YSI 2700, USA) every 5 min. Measured signals of glucose concentration, weight of fermentation broth, pH and DO were connected to a PCLD-8115 wiring terminal board, connected to a PCL-818L data acquisition card. The control signal calculated by the GENIE program was connected as output to a PCLD-885 power relay board. This assembly controlled the pumps connected to glucose (stock concentration as 300 g/l), NaOH (5 N) and HCl (5 N) tanks and also the solenoid valve connected to pure oxygen cylinder. The pH was maintained above 5.4 (by adding NaOH) and below 5.6 (by adding HCl) unless otherwise specified. The control algorithm was designed so that the glucose pump would not pump the calculated amount of glucose to meet the glucose set point (1 or 0.1 g/l), until the pH satisfied the following rule: pH raised, due to the metabolic activity of yeast, and been within the range of 5.51–5.60. DO was controlled above 20% saturation.

2.4. Analytical methods for fermentation process

Total cell concentration was determined by measuring the optical density at 600 nm (OD_{600}) of the fermentation broth. One unit of OD_{600} was found to be equivalent to 0.748 g/l dried cell weight. Cell growth parameters were estimated by non-linear regression of the modified Gompertz model [25] as follows:

$$X = A \exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\}$$

where X is the cell dry weight (g/l) at culture time t (h); A, the maximum cell dry weight (g/l); μ_m, the maximum cell growth rate (g/l per h); λ, the lag time of cell growth (h); and e is the natural logarithm base. The plasmid

stability of recombinant yeast was determined by the fraction of plasmid-containing cells in the replica plating [22] from selective YNBD plates to nonselective YPD plates incubated for 48–72 h at 30 °C. Off-line analysis of ethanol, glycerol and acetate concentrations were determined with a Waters HPLC chromatograph using a Supelcogel C610-H (30 cm × 7.8 mm) column and a Waters 410 RI detector.

2.5. Quantitative analysis of typsin inhibitory activity

The intracellular typsin inhibitory activity of crude sporamin extract from recombinant yeast was measured by first collecting the cells by centrifugation, then disrupting the cells in protein extracting buffer (pH 8.0, 50 mM Tris–HCl and 150 mM NaCl) with a microfluidiser (Microfluidics, USA) operated at 550 kPa. The cell fragments were then removed by centrifugation at 10 000 rpm for 10 min and crude protein extract was collected and the protein concentration estimated by the dye-binding method using Bio-Rad reagent and bovine serum albumin as a standard. To measure sporamin activity, 0.02 ml crude protein extract was incubated in 1 ml reaction buffer (pH 8.0, 50 mM Tris–HCl and 50 mM NaCl) and 0.02 ml trypsin (Sigma, EC 3.4.21.4, type: IX; 0.1 mg/ml in reaction buffer) for 10 min at 37 °C, then 0.01 ml BAPA (50 mg/ml *N*-benzoyl-DL-arginine-4-nitroanilide hydrochloride dissolved in dimethylsulphoxide) was added, and the optical density of the reaction mixture was then measured at 405 nm, continued for 10 min by spectrophotometer (V-500 UV/VIS, Jasco, Japan). Inhibition units, based on 1 U as the complete inhibition of 1 μg trypsin by sporamin extract [27], were calculated using the equation:

$$\text{inhibition units} = \frac{(T - T^*)\alpha}{T}$$

where T denotes the changing slope of ΔOD_{405} per min in the absence of sporamin, T^* indicates the changing slope in the presence of sporamin, and α denotes the α μg of trypsin in the reaction system.

3. Results and discussion

For industrial production with recombinant yeast, the most important problem is maintaining a high concentration of cells carrying recombinant plasmids. Cells with high plasmid stability produce a high specific activity of recombinant protein, i.e. high specific trypsin inhibitory activity (U/g-cell) of sporamin extracted from *S. cerevisiae* KCl in this study. A low concentration of cells with high plasmid stability will produce low total trypsin inhibitory activity (U/l fermentation broth) of recombinant protein, which is of little use. In addition, the cost of medium is another major concern in a commercial process. Based to the plasmid structure of pYE8 cloned in *S. cerevisiae* KCl, the *trp1* gene was used as a selective marker to repress the growth of plasmid-free cells in the selective medium without supplying any amino acid. All the amino acids were, therefore, synthesised from ammonium sulphate in the selective YNBD medium. Therefore, YNBD medium is an expensive medium due to the composition of yeast nitrogen base without any amino acids. Much cheaper complex nitrogen sources, e.g. yeast extract or peptone, were used in this study as in nonselective YPD and YPAD medium. The existence of rich amino acids in the complex nitrogen source will promote the growth of plasmid-free cells due to the segregation instability, but the plasmid stability still depends on the interaction of genetic factors and environmental factors. Zhang et al. concluded that genetic factors include plasmid makeup, copy number, expression level, selective markers and properties of host cells, whereas environmental factors include medium formulation, dissolved oxygen tension, temperature, dilution rate and bioreactor operation modes [7]. A 2 μm-fragment-based plasmid, similar to the plasmid constructed in this study, was shown to help plasmid stability in nonselective medium under proper bioreactor operation modes [26,7].

Table 1 shows a comparison of cell growth conditions and trypsin inhibitory activity on selective YNBD and nonselective YPD medium of different YE content in shake flask culture. It is clear that without proper control of the shake flask culture, the specific inhibitory activity in YPD media were much lower compared with the specific activity in YNBD medium, although comparing the total activity, especially between YNBD and YPD of 1.0% YE, the difference was not so clear. This was due to the much higher growth rate ($\mu_m = 0.298$ g/l per h), higher maximum cell concentration ($A = 4.9$ g/l) and lower lag time ($\lambda = 7.2$ h) on YPD (1.0% YE) medium comparing the growth conditions ($\mu_m = 0.085$ g/l per h, $A = 2.2$ g/l and $\lambda = 32$ h) on YNBD medium.

Therefore, batch fermentation with control of pH, temperature, aeration and agitation was performed, and the cell dry weight (CDW), plasmid stability, specific trypsin inhibitory activity, total inhibitory activity, glucose and ethanol concentration were monitored in YNBD medium (Fig. 1) and YPD (1.0% YE) medium (Fig. 2). All cultures were inoculated identically and as shown in Figs. 1 and 2, both media had very similar initial plasmid stability as well as the specific trypsin inhibitory activity. However, as time proceeded, the cells on the nonselective YPD medium had faster decreasing specific activity as well as faster decreasing plasmid stability, compared with the much higher plasmid stability on selective YNBD medium. The slower decreasing specific activity on YNBD medium was due

Table 1
Cell growth parameters estimated by modified Gompertz model and trypsin inhibition activity on different mediums in shake flask experiments

Medium	λ (h)	μ_m (g/l per h)	A (g/l)	Specific activity (U/g-cell)[a]	Total activity (U/l)[a]
YNBD	32	0.085	2.2	131	302
YPD (0.5% YE)	6.7	0.263	5.3	36	205
YPD (1.0% YE)	7.2	0.298	4.9	50	267
YPD (1.5% YE)	6.9	0.289	4.9	39	205

[a] Maximum total activity at 73 h for YNBD and 52 h for YPD medium.

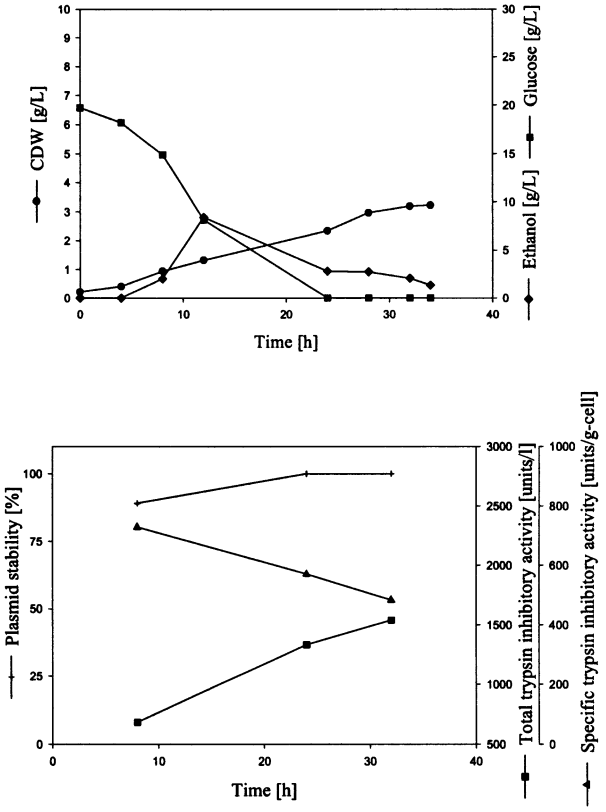

Fig. 1. Cell growth, plasmid stability and typsin inhibitory activity produced by recombinant *S. cerevisiae* KCl on selective YNBD medium in batch fermentation.

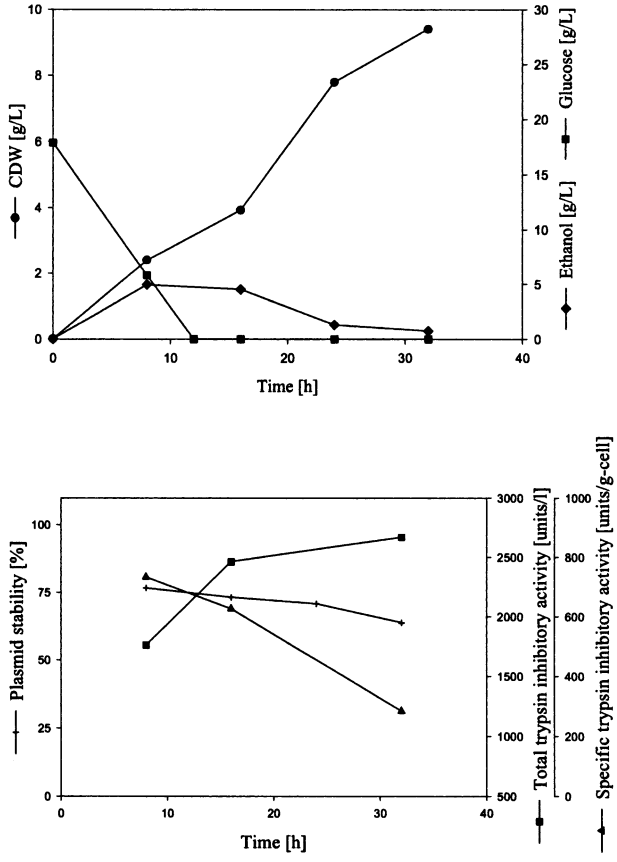

Fig. 2. Cell growth, plasmid stability and trypsin inhibitory activity produced by recombinant *S. cerevisiae* KCl on nonselective YPD (1% YE) medium in batch fermentation.

to dead cell accumulation on cell dry weight, which was used as denominator to calculate the specific activity. Again, cell growth on YPD medium (Fig. 2) had much faster glucose consumption and higher cell concentration, which resulted in much higher total activity compared with growth on YNBD medium (Fig. 1). Ethanol concentrations increased initially until they were consumed as a carbon a source for cell growth after glucose was exhausted in both media.

Table 2 summarises the cell growth conditions and trypsin inhibitory activity in batch fermentations on three different media. The YPAD medium included a simple nitrogen source, ammonium sulphate, to replace part of the complex nitrogen source of peptone in the YPD medium. The ammonium sulphate slightly repressed cell growth and resulted in lower growth conditions ($\mu_m = 0.41$ g/l per h, $A = 7.7$ g/l and $\lambda = 8.5$ h) on YPAD medium compared with better growth conditions ($\mu_m = 0.5$ g/l per h, $A = 9.5$ g/l and $\lambda = 6.0$ h) on YPD medium (Table 2). However, the highest specific inhibitory activity (344 U/g-cell) on YPAD medium compensated for the loss of cell growth and resulted in higher total inhibitory activity (2923 U/l). Table 2 also illustrates that the highest specific activity (463 U/g-cell) in the selective YNBD medium can only result in a much lower total inhibitory activity (1574 U/l) due to the lower cell growth ($\mu_m = 0.12$ g/l per h and $A = 3.4$ g/l), which is clearly of little use. In addition, the

Table 2
Cell growth parameters estimated by a modified Gompertz model and trypsin inhibition activity on different media in batch fermentation

Medium	λ (h)	μ_m (g/l per h)	A (g/l)	Specific activity (U/g-cell)[a]	Total activity (U/l)[a]
YNBD	3.2	0.12	3.4	463	1574
YPD (1.0%YE)	6.0	0.50	9.5	284	2667
YPAD	8.5	0.41	7.7	344	2923

[a] Maximum total activity at 32 h.

cost of YPAD medium is the lowest of the three media (Table 2).

Recombinant yeast growth on the selective YNBD medium always produced higher specific trypsin inhibitory activity than cell growth on a nonselective medium under crude-control fermentation conditions such as shake flask or batch fermentation (Tables 1 and 2). Therefore, it was necessary to look for a better (fed-batch) fermentation control strategy, apart from the further improvement of medium composition in batch culture. Several methods have previously been proposed to improve plasmid stability under nonselective conditions for recombinant *S. cerevisiae* [11–24]. Their major goal was developing a control strategy for substrate starvation to help less efficient growth of plasmid-containing cells to catch up to the more efficient growth of plasmid-free cells. However, the crude-control of carbon source concentration usually resulted in low cell growth rate or the accumulation of ethanol, glycerol and acetic acid, which inhibited the expression of recombinant protein [16,18,20,21,23,24].

The glucose/pH control strategy can maintain high specific trypsin inhibitory activity, while maintaining a high cell growth rate to reach high total trypsin inhibitory activity during fed-batch fermentation on the nonselective YPAD medium (Fig. 3). Fig. 3(a) shows that when the controller set point of glucose concentration was kept at 1.0 g/l, the specific activity declined slightly; but the specific activity was maintained much better when the glucose set point was kept at 0.1 g/l, as shown in Fig. 3(b). Table 3 summarises the cell growth conditions and trypsin inhibitory activity in fed-batch fermentations based on different glucose set points. It indicates that the set point of low glucose concentration slightly represses cell growth and results in a lower growth rate ($\mu_m = 0.5$ g/l per h) compared with the rate ($\mu_m = 0.57$ g/l per h) at the 1.0 g/l glucose set point. However, the much higher specific inhibitory activity (831 U/g-cell) at 0.1 g/l glucose set point compensated for the loss of growth rate and resulted in a significant increase of total inhibitory activity (12 222 U/l). A much lower concentration of ethanol, acetic acid and glycerol was maintained during the glucose controlled set point at 0.1 g/l comparing to Fig. 4(a), with the set point at 1.0 g/l.

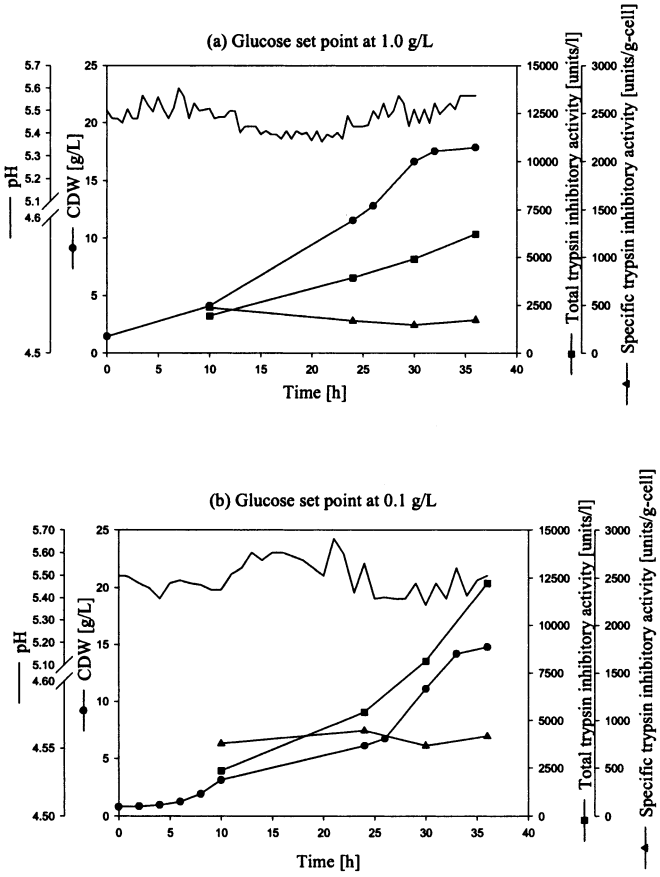

Fig. 3. Comparison of cell growth and trypsin inhibitory activity produced by recombinant *S. cerevisiae* KCl on nonselective YPAD medium controlled with different set-points of glucose concentration in fed-batch fermentation.

The on-line measured data of glucose concentration in Fig. 4(a) shows that because the control strategy was designed so that the glucose feeding action was inactive until the pH was within 5.51–5.6, then the glucose concentration was controlled close to zero (even the glucose set point at 1.0 g/l) during 17–24 h fermentation time (refer to the detail pH profile in Fig. 3(a)). After 24 h the glucose concentration was controlled within 0.5–1.3 g/l, and then the accumulation of ethanol, acetic acid and glycerol increased compared with the complete repression of those metabolites in Fig. 4(b), with glucose set point at 0.1 g/l. Therefore, the low glucose concentration, which produces carbon source starvation and

Table 3
Cell growth parameters estimated by a modified Gompertz model and trypsin inhibition activity under different glucose set points on YPAD medium in fed-batch fermentation

Medium	λ (h)	μ_m (g/l per h)	A (g/l)	Specific activity (U/g-cell)[a]	Total activity (U/l)[a]
1.0	4.4	0.57	20.5	347	6212
0.1	9.0	0.50	20.2	831	12 222

[a] Maximum total activity at 36 h.

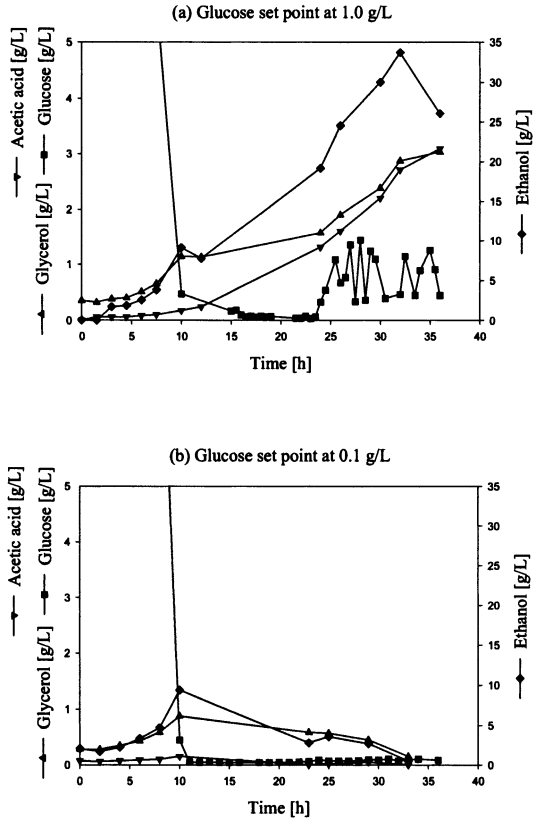

Fig. 4. Comparison of glucose, ethanol, acetic acid and glycerol concentration profiles during fed-batch fermentation, as controlled with different set points of glucose concentration for recombinant *S. cerevisiae* KCl on nonselective YPAD medium.

low concentration of ethanol, acetic acid and glycerol maintained in the controlled conditions of Fig. 3(b), Fig. 4(b), offers an excellent environment for the plasmid-carrying yeast to compete with the plasmid-free yeast and express the recombinant sporamin without metabolite repression.

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