Bioprocess Biosyst Eng (2014) 37:1887–1898
DOI 10.1007/s00449-014-1163-z

ORIGINAL PAPER

Simplified feeding strategies for the fed-batch cultivation of *Kluyveromyces lactis* GG799 for enhanced recombinant xylanase production

Siti Fatimah Zaharah Mohamad Fuzi · Firdausi Razali · Jamaliah Md. Jahim · Roshanida A. Rahman · Rosli Md. Illias

Received: 31 October 2013 / Accepted: 26 February 2014 / Published online: 15 March 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract A xylanase gene (*xyn2*) from *Trichoderma reesei* ATCC 58350 was previously cloned and expressed in *Kluyveromyces lactis* GG799. The production of the recombinant xylanase was conducted in a developed medium with an optimised batch and with fed-batches that were processed with glucose. The glucose served as a carbon source for cell growth and as an inducer for xylanase production. In a 1-L batch system, a glucose concentration of 20 g L^{-1} and 80 % dissolved oxygen were found to provide the best conditions for the tested ranges. A xylanase activity of 75.53 U mL^{-1} was obtained. However, in the batch mode, glucose depletions reduced the synthesis of recombinant xylanase by *K. lactis* GG799. To maximise the production of xylanase, further optimisation was performed using exponential feeding. We investigated the effects of various nitrogen sources combined with the carbon to nitrogen (C/N) molar ratio on the production of xylanase. Of the various nitrogen sources, yeast extract was found to be the most useful for recombinant xylanase production. The highest xylanase production (110.13 U mL^{-1}) was measured at a C/N ratio of 50.08. These conditions led to a 45.8 % increase in xylanase activity compared with the batch cultures. Interestingly, the further addition of 500 g L^{-1} glucose led to a 6.2-fold increase (465.07 U mL^{-1}) in recombinant xylanase activity. These findings, together with those of the exponential feeding strategy, indicate that the composition of the C/N molar ratio has a substantial impact on recombinant protein production in *K. lactis*.

Keywords Recombinant xylanase production · *K. lactis* optimisation · C/N molar ratio · Feeding strategy

S. F. Z. M. Fuzi
Technology and Heritage Department, Faculty of Science, Technology and Human Development, Universiti Tun Hussein Onn, 86400 Parit Raja, Batu Pahat, Johor, Malaysia

F. Razali · R. A. Rahman · R. Md. Illias (✉)
Bioprocess Engineering Department, Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
e-mail: r-rosli@utm.my

J. Md. Jahim
Chemical and Process Engineering Department, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

Introduction

In comparison with bacterial systems, yeast possesses a number of attributes that render it an attractive expression platform for many recombinant proteins [1]. In particular, the yeast *Kluyveromyces lactis* has attracted industrial interest as an alternative host for the secretion of heterologous proteins on a large scale [2]. Indeed, *K. lactis* has produced over 40 recombinant proteins, and it is best known for its use in the commercial production of bovine chymosin [3]. The capability of *K. lactis* to grow on a variety of inexpensive carbon sources while efficiently secreting extracellular proteins represents the major strength of this system [2].

Most production using recombinant *K. lactis* has been conducted in batch processes [3, 4]. However, nutrient limitation and starvation often lead to "stuck" batch fermentation. The most prominent effect of this phenomenon is reduced cell growth, whereby the production of recombinant proteins causes significant alterations to the central carbon metabolism of the host [5]. This in turn leads to the

redirection of the carbon source from biomass or by-product formation towards energy formation.

Likewise, limiting the carbon or nitrogen sources or the dissolved oxygen (DO) concentration may influence the physiological state of the yeast, potentially affecting its cell growth, protein secretion and product formation [6]. Even the composition, synthesis and maintenance of the cell wall respond to environmental changes [7]. Hsieh and Da Silva [8] observed that glucose is an important inducer of recombinant enzyme production and is also important in providing carbon, generating energy and allowing for cell structure and maintenance. In contrast to glucose, nitrogen depletion results in an increase in protease activity, which can be avoided by increasing initial nitrogen concentrations. Protein yields were shown to improve in several cultures grown on complex or amino acid-enriched media [9]. Complex or enriched media not only prevent nutrient limitation but may also inhibit protease activity by providing competing enzymatic substrates. Thus, the optimisation of the C/N molar ratio is important, as it has a strong effect on yeast metabolism, influencing its channelling towards cell growth, protein synthesis or the production of secondary metabolites.

Therefore, the use of fed-batch fermentation can alleviate commonly encountered problems such as substrate inhibition, low cell concentrations, glucose effects, catabolite repression, auxotrophic mutants and the high viscosity of the culture broth [10]. A number of studies have addressed the optimisation of fermentation conditions to achieve higher yields of heterologous proteins using *K. lactis* [11–13]. These authors have studied the importance of factors such as pH, lactose concentration and temperature during fed-batch fermentation. However, the information provided in such studies has been insufficient to develop a process for recombinant *K. lactis* production using fed-batch cultures.

The full-length *xyn2* gene from *Trichoderma reesei* ATCC 58535 has been successfully expressed and secreted in *K. lactis* GG799 using shake-flask cultures. The *xyn2* gene was cloned into integrative vector pKLAC1, located downstream of the *K. lactis* α-mating factor (αMF) signal sequence. The constructed pKLAC1*xyn2* vector was linearised with *Sac*II before being integrated into the *K. lactis* genome at the *LAC4* promoter locus. Thus, the expression of xylanase is under the control of the *LAC4* promoter in this system. The transformation of *K. lactis* GG799 was achieved by a chemical method, as described elsewhere [14].

In this study, the effects of exponential feeding and C/N ratio optimisation on the feed stream were investigated. The pKLAC1*xyn2* regulated fed-batch cultivation of *K. lactis* GG799 is divided into two phases: the glucose batch and glucose–yeast extract fed-batch phases. To this end, an improved strategy for controlling this composition was developed, which was designed to improve recombinant xylanase production. However, an unlimited glucose supply can lead to oxygen depletion, and oxygen limitation can negatively affect protein expression [15, 16]. Moreover, the DO concentration is a critical parameter for high cell density cultivation [17]. Therefore, before fed-batch fermentation was conducted, the initial glucose concentration and DO level were optimised under batch fermentation. The results were analysed using a three-dimensional response surface plot. The objective of this study was to determine the optimal steps required to produce the most xylanase in the shortest period of time. These steps are as follows: (1) establishing batch fermentation with the optimal conditions for the tested range, (2) determining a good nitrogen source for feeding and (3) performing exponential fed-batch fermentation using various C/N molar ratios. To the best of our knowledge, this study is the first to employ an exponential feeding strategy to improve the expression of recombinant enzymes in *K. lactis* under the control of *LAC4* in optimised oxygen level conditions.

Materials and methods

Strain and inoculum development

The *xyn2* gene was cloned into integrative vector pKLAC1, as mentioned in the "Introduction". The recombinant yeast *K. lactis* GG799 (*K. lactis* Protein Expression Kit, New England Biolabs, Inc., Ipswich, MA) was used throughout the study and was maintained on yeast extract peptone (YPD) slant agar (20 g glucose, 20 g peptone, 10 g yeast extract and 15 g L^{-1} agar) at 4 °C.

The inoculum culture was prepared from cells grown on YPD slant agar suspended in 100 mL YPD broth (glucose 20 g, peptone 20 g and yeast extract 10 g L^{-1}). The cells were incubated at 30 °C ± 0.5 °C on a rotary shaker at 250 rpm until their growth reached the mid-exponential phase [i.e., an A_{660} (absorbance of 660 nm) of 1.7]. The cultures were then centrifuged at 6,000 rpm for 10 min and washed twice with sterile distilled water. The cell pellets were resuspended to a volume of 100 ml prior to being used in the batch fermentation. A 10 % *K. lactis* GG799 inoculum, with an A_{660} of 5–6, was used throughout the study unless otherwise mentioned. All chemicals were purchased from Difco Becton–Dickinson (USA), Sigma-Aldrich (USA) and Merck (Germany) unless otherwise stated.

Production medium

Batch and fed-batch fermentation was conducted in a medium composed of 5 g $(NH_4)_2SO_4$, 1.69 g $MgSO_4{\cdot}7H_2O$,

0.18 g Na_2SO_4, 3.93 g casamino acid, 0.24 g $ZnSO_4{\cdot}6H_2O$, 1.00 g $MnSO_4{\cdot}4H_2O$, 7.66 g L^{-1} KH_2PO_4 and glucose and buffered to a pH of 5.0 ± 0.2 using a 50-mM phosphate–citrate buffer [14]. A B. Braun bioreactor (Biostat B model, B. Braun Biotech International, Germany) was employed for the fermentation process. The working volume for batch fermentation was 1 L. For fed-batch fermentation, a mixture of 400 g L^{-1} glucose and a nitrogen source was fed into the batch after the initial depletion of glucose to 20 g L^{-1}. To maintain the glucose level above 20 g L^{-1} in the culture broth, the feed rate was changed as described in the Section "Results and discussion".

Submerged cultivation in the bioreactor

The temperature was 28 °C, and the initial pH was 5.0 ± 0.2, as regulated by 2 M HCl and 2 M Na_2CO_3. The pH was measured using a sterilisable electrode (Ingold model, Mettler-Toledo, Alphaville-Barueri, Brazil), and the DO concentration was detected using a polarographic electrode (Ingold model, Mettler-Toledo, Alphaville-Barueri, Brazil). During the experiments, oxygen was supplied by sparging the bioreactor with compressed air at an agitation frequency of 300–800 rpm until the desired DO level was reached. In the batch fermentation, the set points for the DO level and glucose concentration were adjusted to meet the process parameters designed using the CCD. After the proper incubation time designated for each set, the samples were collected, and the cell-free supernatant was obtained by 10 min of centrifugation at 10,000 rpm and 4 °C. The cell-free supernatant was used to determine the production levels of enzymes and other metabolites.

The initial feed rate was calculated from the mass balance on the substrate according to the following formula, as described by Wang et al. [18]:

$$F = \frac{\mu (XV)_0 e^{\mu t_f}}{Y_{x/sf}} \tag{1}$$

where μ is the specific growth rate, t_f is the time at the start of feeding, F is the feed rate, XV_0 are the biomass concentration (gdw L^{-1}) and the culture volume (L) at the start of feeding, respectively, S_f (g L^{-1}) is the substrate concentration in the feeding solution, and $Y_{x/sf}$ is the yield coefficient (g dry cell weight g of glucose^{-1}).

Factorial design and statistical analysis

Response surface methodology (RSM) was used to examine the influence of the initial glucose concentration and of the DO level on the activity of the recombinant xylanase and the cell growth, as well as to determine the optimum fermentation conditions. Software by Design Expert (Statistics Made Easy, v.6.0.4, Stat-Ease Inc., Minneapolis, MN, USA, 2001) was used to generate the experimental design. The RSM CCD (central composite design) was used to obtain data that fit a full second-order polynomial model. A 2^2 factorial design with axial points ($\alpha = \sqrt{2}$) and five replicates at the central point was employed. Thirteen experiments were required for this procedure, as shown in Table 1. Regression analysis (using ANOVA) was employed to determine the significance of the second-order model and to subsequently generate the response surface graphs. Triplicate experiments were run using the optimum values for the variables, as determined by Eq. (2).

$$Y_i = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j \tag{2}$$

where Y is the predicted value for the response, x_i and x_j are the design variables, β_0 is the intercept estimate, and β_i is the estimate of the unknown coefficient for the ith factor.

Analytical determinations

The xylanase activity in the cell-free supernatant was assayed using the modified dinitrosalicylic acid (DNS) method [19] at 50 °C with 0.01 g L^{-1} of beechwood xylan (Sigma) as the substrate. The DNS method was performed as follows: 1.0 g of xylan was homogenised in approximately 80 mL buffer at 45 °C and heated to its boiling point on a heating magnetic stirrer [19]. The mixture was cooled with continued stirring, covered and stirred slowly overnight. Its volume was increased to 100 ml with buffer. Appropriate dilutions of the supernatant in 50 mM sodium citrate buffer (pH 5.0 ± 0.2) were used as the enzyme source. The amount of enzyme was determined by measuring the release of reducing sugars. One unit of xylanase was defined as the amount of enzyme required to liberate 1 μmole of xylose from xylan per minute under assay conditions.

The biomass concentration was determined by turbidity measurements at an A_{660} using a UV/visible spectrophotometer (Ultrospec 1100 Pro; Amersham Bioscience, Sweden). The measured values were correlated with the dry weight values of duplicate samples [14]. The factor used to calculate dry mass is as follows:

$$\text{dry mass}\left(\text{g L}^{-1}\right) = 0.379 \times A_{660}$$

The glucose concentration was determined using the DNS method [20]. Briefly, 750 μL of DNS reactive agent was added to a 750-μL supernatant sample, which had been diluted to decrease the glucose concentration to <2 g L^{-1}. Tubes containing the reaction mixture were heated in a boiling bath for 15 min and then cooled. The final solution absorbance was read at 540 nm, and the glucose concentration was calculated using a glucose standard curve.

Ethanol was measured using a SRI 8610C capillary column (30 m long, with a 0.25-mm inside diameter and a

Table 1 Experimental design used in the RSM studies

Run no. (Code level)	Levels	Variables: DO level A (%) Axial points (±α)	Variables: Glucose concentration (g L^{-1}) B Centre point (0)	Responses: Xylanase activity (U mL^{-1}) Factorial points (±1)	Responses: Biomass concentration (g L^{-1})
1		37 (−1)	13 (−1)	40.38	3.84
2		55 (0)	20 (0)	41.96	4.19
3		55 (0)	30 (1.412)	34.77	4.37
4		30 (−1.412)	20 (0)	43.54	4.08
5		37 (−1)	27 (1)	38.4	3.96
6		55 (0)	20 (0)	50.07	4.06
7		55 (0)	10 (−1.412)	19.25	3.96
8		73 (1)	27 (1)	54.53	4.44
9		73 (1)	13 (−1)	48.27	4.07
10		55 (0)	20 (0)	48.66	4.02
11		55 (0)	20 (0)	42.77	4.12
12		80 (1.412)	20 (0)	75.30	4.42
13		55 (0)	20 (0)	47.07	4.10

In the table above, the rows represent individual experiments and the columns denote the actual values of the treatment combinations and determined responses. The numbers in the parentheses represent the coded values of the DO level and the initial glucose concentration

Two variables, the DO level and the initial glucose concentration, were altered, and the recombinant xylanase activity and biomass concentration were measured. High and low values are chosen for each variable. The initial ranges of the variables are as follows: the DO level varied from 30 to 80 % and the initial glucose concentration from 10 to 30 g L^{-1} [14]

film thickness of 0.25 μm) equipped with a flame ionisation detector. Ethanol was quantified using external standards, with 2-propanol as the internal standard. Each sample was injected three times to assure its reproducibility (the variation coefficient was <6 %).

The total protein concentration was quantified by the Bradford method [21], using bovine serum albumin as the standard protein.

All analyses were performed in duplicate. The data presented represent the measurements taken, and all standard errors were <5 %.

Kinetics studies

The logistic model employs rate equations for the *K. lactis* GG799 biomass (*X*) and the amounts of recombinant xylanase (*P*) and glucose (*S*) to describe the fermentation process. Equation (3) describes the rate of change of the *K. lactis* GG799 biomass:

$$dX/dt = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X. \quad (3)$$

The kinetics of product formation was based on the Leudeking–Piret equation, as described by Eq. (4):

$$dP/dt = \alpha(dX/dt) + \beta X \quad (4)$$

The glucose consumption equation is a modified Luedeking–Piret equation [22], as described by Eq. (5):

$$-dS/dt = \gamma dX/dt + \delta X \quad (5)$$

where X is the cell concentration (g L^{-1}), X_{max} is the maximum cell concentration (g L^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), S is the substrate concentration (g L^{-1}), α is the growth-associated constant for product formation (U g $cell^{-1}$), β is the non-growth-associated constant for product formation (U g $cell^{-1}$), γ is the growth-associated constant for substrate consumption (g substrate g $cell^{-1}$), δ is the non-growth-associated constant for substrate consumption (g substrate g $cell^{-1}$ h^{-1}), and t is the fermentation time (h). The kinetics models (Eqs. 3–5) were fitted to the experimental data by nonlinear least squares regression using SigmaPlot software, version 10 (Systat Inc., USA), to solve for the unknown kinetics parameters. The predicted ideal values were then used to simulate the profiles of the cells and the substrate consumption during fermentation.

Calculation of the C/N ratio

The C/N ratio was calculated on a molar basis. The formula used for the conversion of the mass concentration of glucose (g L^{-1}) to the mM concentration of carbon was [glucose (g L^{-1}) × 33.3 = mM carbon]. The conversion of the yeast extract mass concentration (g L) to the mM concentration of nitrogen was [yeast extract (g L^{-1}) × 11.4 = mM nitrogen], and the conversion of the $(NH_4)_2SO_4$ mass concentration (g L^{-1}) to the mM concentration

of nitrogen was [$(NH_4)_2SO_4$ (g L^{-1}) × 15.2 = mM nitrogen].

Results and discussion

Optimisation of recombinant xylanase and biomass production by *K. lactis* GG799 in a 2-L bioreactor: construction of the polynomial model for batch fermentation

Using a CCD, experiments with different combinations of the DO level and initial glucose concentration were performed to determine their effects on the production of recombinant xylanase and the biomass yield. Table 1 presents a matrix of the complete CCD that shows the activity obtained after 6 h of fermentation for all the different conditions. A fermentation time of 6 h was chosen based on previous studies that were performed by the same group in a shake-flask cultivation system [14]. The minimum activity of recombinant xylanase was 19.25 U mL^{-1}, while the maximum activity was 75.30 U mL^{-1}, as shown in Table 1. This high value was due to the optimised conditions for enzyme activity. However, no significant differences were observed for the biomass concentration: the minimum biomass concentration was 3.84 g L^{-1}, while the maximum biomass concentration was 4.44 g L^{-1}. These results also suggest an interaction between the cell density and oxygen concentration. The findings reveal that the production of recombinant xylanase was at its maximum at a DO level of 80 % and a glucose concentration of 20 g L^{-1}. Under these conditions, an activity of 75.3 U mL^{-1} was obtained.

The ANOVA model for recombinant xylanase production in *K. lactis* GG799 has a coefficient of determination (R^2) of 0.9191, which indicates that 91.91 % of the variability in the data can be explained effectively. The fit of the cell concentration model was also expressed by an R^2 value, which showed that 78.85 % of the observed response could be explained by the model. These relatively high R^2 values indicate that both the linear and the second-order polynomials are capable of representing the system under the given experimental design [23].

During the batch fermentation, the specific growth rate, μ, varied between 0.06 and 0.13 h^{-1} (Table 2). Boender et al. [24] stated that a doubling time of 23.1 h^{-1} represents extremely fast growth for yeast. Thus, during this study, the recombinant yeast *K. lactis* GG799 grew rapidly under most conditions because the doubling times during cultivation were between 6 and 11 h $\left(T_d \frac{\ln 2}{\mu}\right)$. Additional analysis showed that the biomass yield $\left(Y_{x/s}\right)$ was between 0.17 g cell g $glucose^{-1}$ and 0.40 g cell g $glucose^{-1}$. These biomass yields were lower than the experimental value of $Y_{x/s} = 0.64$ g cell g $glucose^{-1}$ that was obtained for untransformed *K. lactis* GG799. This result suggests that recombinant xylanase production in *K. lactis* GG799 involves a disturbance in its cellular metabolism, which can be observed in its decrease in growth rate and biomass yield. This pattern was especially evident under conditions of 80 % DO and 20 g L^{-1} glucose. Under these conditions, the increase in the xylanase activity (75.30 U mL^{-1}) corresponded with a decrease in the yeast growth rate and biomass, as shown in Table 2 (at 0.09 h^{-1} and 0.17 g cell g $glucose^{-1}$, respectively).

Validation of the model through recombinant xylanase production and cell growth under optimised conditions

According to the mathematical model predicted by SigmaPlot, the optimal conditions for the tested range of the two variables were a DO level of 80 % and a glucose concentration of 20 g L^{-1}. These values would theoretically yield a corresponding maximum recombinant xylanase activity of 72.70 U mL^{-1} and a biomass of 4.33 g L^{-1}. As shown in Fig. 1, the recombinant xylanase production exhibited a peak of 75.53 U mL^{-1} and a biomass production peak of 4.10 g L^{-1} after 6 h of incubation. As

Table 2 Comparison of the performance and kinetic parameter values of the recombinant xylanase production in the batch fermentations of *K. lactis* GG799 using a defined medium with differing DO levels and glucose concentrations

Kinetic parameter values	DO level (%)*; initial glucose concentration (g L^{-1})								
	37; 13	55; 20	55; 30	30; 20	37; 27	55; 10	73; 27	73; 13	80; 20
Recombinant xylanase activity (U mL^{-1})	40. 38	41.96	34.77	43.54	38.4	19.25	54.53	48.27	75.3
Maximum biomass produced, X_{max} (g L^{-1})	4.80	6.47	6.12	5.38	6.09	4.91	7.07	5.54	6.33
Maximum specific growth rate, μ_{max} (h^{-1})	0.11	0.12	0.09	0.12	0.06	0.10	0.13	0.07	0.09
Yield, $Y_{x/s}$ (g substrate g $cell^{-1}$)	0.31	0.40	0.25	0.24	0.22	0.30	0.35	0.32	0.17
Maximum ethanol production (g L^{-1})	0.09	0.08	0.02	0.17	0.58	0.36	0.07	0.36	0.02
Productivity (U mL^{-1} h^{-1})	6.73	6.99	5.80	7.26	6.4	3.21	9.09	8.05	12.55

* These ratios were obtained from preliminary experiments (not shown)

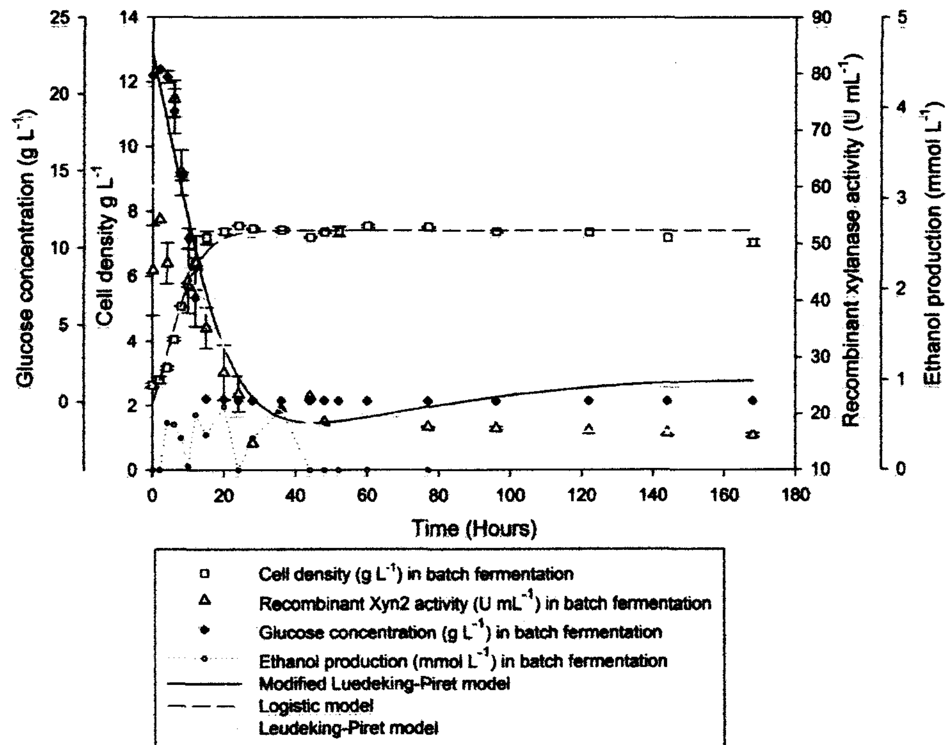

Fig. 1 Recombinant *K. lactis* GG799 cultivation using batch fermentation under optimal conditions for the tested range: simulation curves and experimental data for the total biomass (*open squares*), glucose (*filled diamonds*) and recombinant xylanase (*filled triangles*). Ethanol (*grey diamonds*) was the by-product of the fermentation. The data shown are derived from triplicate means. The parameters used for the simulation were as follows: $\mu_{max} = 0.08\ h^{-1}$, X_{max}= 7.56 g L^{-1}, $Y_{x/s}$= 0.16 g substrate g $cell^{-1}$ and $m_s = 0.011$ g substrate g $cell^{-1}\ h^{-1}$

described by Sawale and Lele [25], a good correlation between the predicted and experimental results verifies the model.

During these tests, the parameters μ_{max}, X_{max}, $Y_{x/s}$ and m_s were experimentally determined. The m_s value was used in the modified Luedeking–Piret model to express the glucose consumption. The sigmoidal growth pattern of *K. lactis* GG799 was analysed using a logistic equation (Eq. 3) for its variation against time (Fig. 1). The equation is a substrate-independent rate model, which was used to determine the inhibition effect on biomass growth. The logistic and Leudeking–Piret equations provided a good description of the cell growth, recombinant xylanase production and substrate consumption versus batch fermentation time in the *K. lactis* GG799 batch production. In the kinetics studies, recombinant xylanase production was assumed to be a growth-associated product, such that the growth-associated constant α is 18.03 U mg^{-1} and the non-growth-associated constant β is neglected.

In heterotrophic organisms, the seemingly lower cell maintenance requirement of 0.011 g substrate g $cell^{-1}\ h^{-1}$ is due to the regulation induced by a stringent response, which results in a linear time-dependent increase in the biomass, as previously reported [26]. In this study, the cells continued to grow and proliferate until the stationary phase was reached. The subsequent halt in cell growth may be due to the depletion of the carbon source and other possible nutrient limitations, such as insufficient nitrogen-containing vitamins. Oxygen no longer appeared to be the primary limiting factor under this experimental condition. However, a small amount of ethanol remained until the end of fermentation (0.1 mmol ml^{-1}). Thus, when the growth rate decreased, the ethanol yield appeared to be related to the cell maintenance.

The highest activity of xylanase was obtained (75.53 U mL^{-1}) under optimised aerobic conditions. This finding contradicts those of Huang and Demirci [11], who stated that conditions without aeration or pH control increased the human lysozyme production in *K. lactis*. This result may be due to the ability of some *K. lactis* strains to ferment and grow efficiently under anaerobic conditions, in contrast with other strains [27, 28]. Merico et al. [29] stated that it is unclear whether the aerobic nature of *K. lactis* is an original trait or a recently derived one. For the recombinant *K. lactis* GG799, both fermentation and respiration were observed; although respiration is predominant in this case, fermentative metabolism is sufficient to allow anaerobic growth. Based on this observation, *K. lactis* GG799 could be characterised as respiro-fermentative yeast. Furthermore, a limited oxygen supply appears to affect the quality of the produced xylanase, which could be due to proteolytic degradation, the incorporation of incorrect amino acids or incorrect posttranslational modifications in the cells.

Fed-batch fermentation

Before the optimisation of the C/N molar ratio, a study on the effect of supplementation with various nitrogen sources on growth and xylanase expression was conducted (Fig. 2a, b). In general, the recombinant xylanase activity in the nitrogen-containing medium declined after 6 h of fermentation. The activity of recombinant xylanase was the highest in early cultivation in medium containing copper–glycine, but slightly lower than the urea-containing medium after 6 h (Fig. 2b). Copper–glycine was intentionally introduced to the cultures to produce a copper-enriched yeast biomass as mentioned by Manzoni et al. [30]. However, the copper concentration must remain low, as Kershaw et al. [31] reported that excess copper–glycine is involved in the superoxide stress response, iron homoeostasis and envelope stress. These stresses will inevitably result in plasmid instability and the deterioration of protein production. Therefore, copper was predicted to not have a significant effect on protein folding within the xylanase GH11 family, which includes *T. reesei* Xyn2 as mentioned by Yi et al. [32]. For this reason, copper–glycine medium was not chosen for the remainder of the experiments. In contrast to other nitrogen-containing media, yeast extract has been demonstrated to improve the culture density of recombinant *K. lactis* GG799 at the end of fermentation (Fig. 2a). When yeast extract is present, carbon, amino acids and inorganic nutrients from yeast extract may overcome the "stuck" phase of the batch fermentation. This therefore represents a good strategy to utilise yeast extract, as it could allow for the synthesis of so-called starvation proteins, which are required for cell survival in the new conditions during the fed-batch process. This strategy is similar to the fed-batch feeding strategy used for recombinant *Pichia pastoris*. In addition, the enzyme maintains 58 % of its maximum activity for up to 12 h of cultivation. Using yeast extract, the percentage of xylanase reduction is equivalent to those of the other nitrogen sources used. The decline of xylanase synthesis, rather than the increased proteolysis, is likely to be the underlying reason for the poorer recombinant xylanase production. This finding suggests the need of a booster factor to be used during fed-batch production with *K. lactis* GG799.

The fed-batch cultivation of *K. lactis* GG799 under the control of *LAC4* is divided into two phases: a 6-h batch phase and a fed-batch phase. In order for the cells to grow at a constant rate, exponential feeding strategies were employed in the fed-batch experiments in which the *F*/*V* ratio was held constant [33]. Effect of various feeding range on biomass production was tested (data not shown). The experiments were carried out with an initial concentration of 20 g L^{-1} glucose and 500 mL glucose solution with medium added at 6 h by an exponential feeding as shown in Eq. (1).

The μ value was obtained from the batch fermentation. Combined with this, the optimisation of the C/N molar ratio with different yeast concentrations was tested in the range of

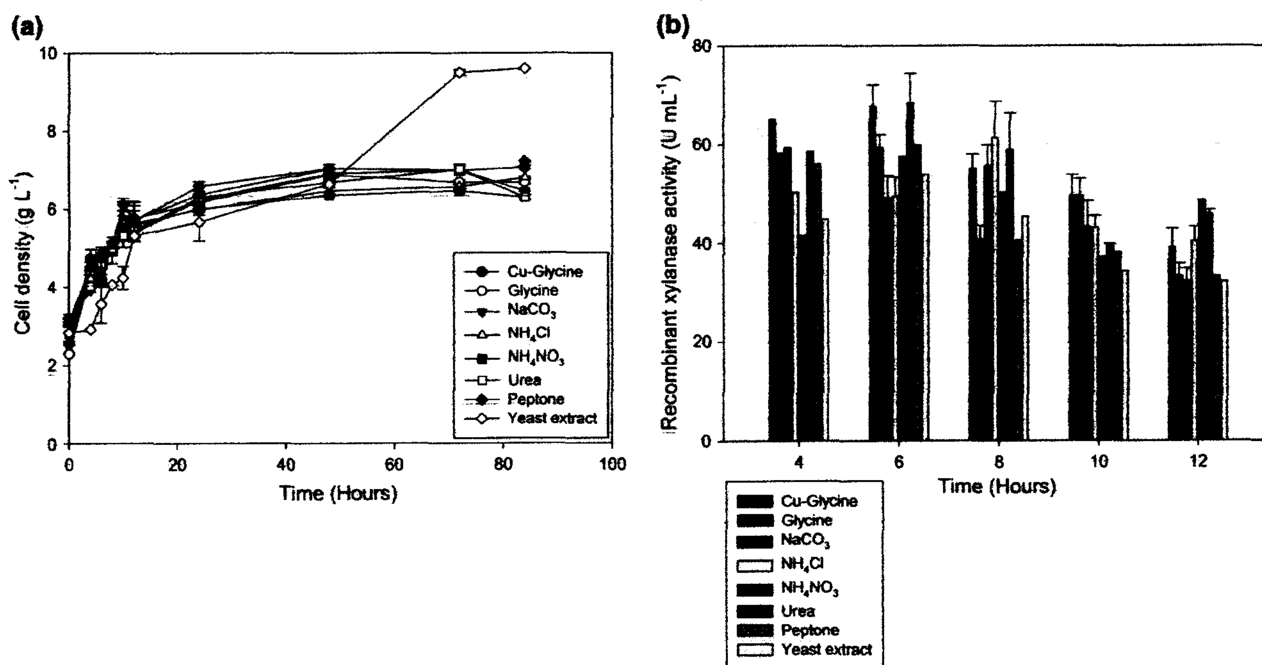

Fig. 2 Effects of eight different nitrogen-source-containing media on **a** the cell growth and **b** the xylanase production of recombinant *K. lactis* GG799. *Each line* represents the biomass concentrations in the media as the mean ± standard error ($n = 3$)

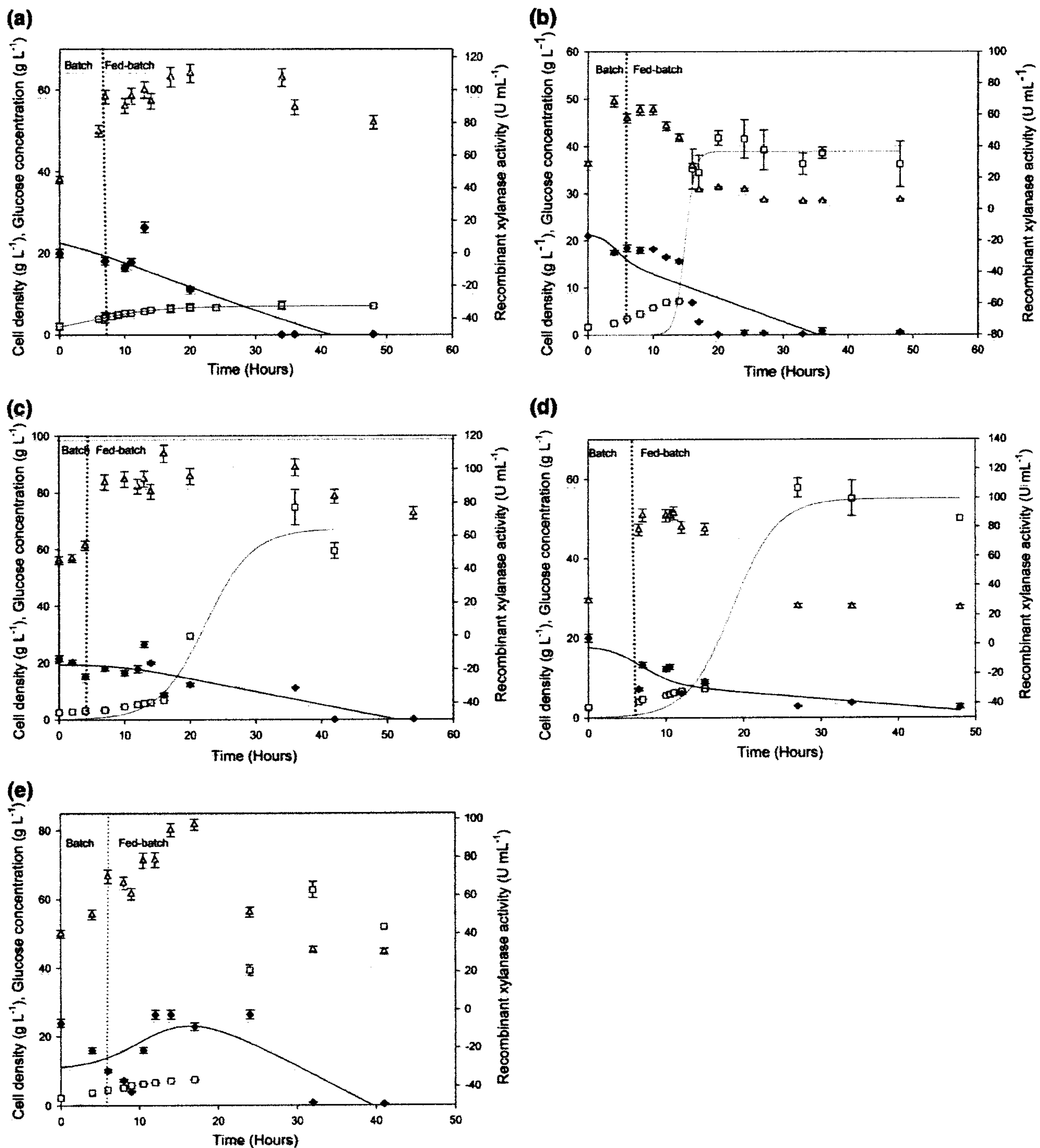

Fig. 3 Time course of recombinant xylanase production by *K. lactis* GG799 in batch and fed-batch fermentation (exponential rate). *Symbols* represent the following: *filed diamonds* recombinant xylanase activity, *filled squares* cell growth, *filled circles* glucose concentration. After 6 h of batch fermentation, the fed-batch was initiated using different ranges of yeast extract concentrations. The feeding medium consists of DM medium with 200 g L^{-1} glucose and **a** 5 g L^{-1} yeast extract, **b** 10 g L^{-1} yeast extract, **c** 20 g L^{-1} yeast extract, **d** 30 g L^{-1} yeast extract or **e** 40 g L^{-1} yeast extract. The *continuous lines* represent model predictions with 5 % deviation

5–40 g L^{-1} (Fig. 3). Experimental results were analysed using SigmaPlot software, and the values for the parameters of α, β and $Y_{x/s}$ are shown in Table 3. Comparisons between the model predictions and the experimental data are given in Fig. 3 as well. The continuous lines are the model values with approximately 5 % deviation.

Table 3 Estimation of the model parameters from experimental data

Parameters	Initial yeast extract concentration (g L^{-1})	Batch			Fed-batch		
		–	5	10	20	30	40
$Y_{x/s}$ (g dry cell weight g of glucose^{-1})		0.17	0.09	2.61	3.49	2.85	2.47
μ_{max} (h^{-1})		0.08	0.12	0.75	0.33	0.45	0.30
α (U g cell^{-1})		18	50	50	50	50	50
β (U g cell^{-1})		0	0	0	0	0	0
γ (g substrate g cell^{-1})		8	27	18	18	17	17
δ (g substrate g cell^{-1})		0.01	0.01	0.01	0.01	0.01	0.01

Parameters for model prediction in batch and fed-batch fermentation

The feeding of 5 g L^{-1} of yeast extract did not increase the growth of *K. lactis* GG799 until the end of the fermentation period (Fig. 3a). However, adding higher concentrations of yeast extract significantly increased the cell density, as described in Fig. 3b–e. This indicated that nitrogen source was the limiting factor for cell growth, but may not the prominent cause of proteolytic activity in *K. lactis* GG799.

An increase in the growth rate was temporarily achieved by the boosting step, and an approximately six- to tenfold increase (41.81–74.85 g L^{-1}) in the cell density occurred within 18 h. To better observe the growth profiles, the biomass production under a range of yeast extract concentrations was plotted against time (Fig. 4).

Our results show that this medium boost significantly improved the cell density. The new system is no longer based entirely on a medium composed of plain mineral salt but that is instead supplemented with yeast extract. The addition of yeast extract at the time of expression provides a sufficient supply of biosynthetic precursors that can be channelled into anabolic pathways and that are required for efficient protein synthesis. The beneficial effects of complex additives have been previously reported in the literature [34]. These authors demonstrated that the supplementation of culture medium with amino acids or complex nitrogen sources resulted in improved levels of recombinant protein expression in *S. cerevisiae.*

A summary of the effects of the yeast extract concentration in the feeding medium on the growth and recombinant xylanase production is shown in Table 4. The addition of 5 g L^{-1} of yeast extract did not improve the biomass concentrations, but did increase the xylanase activity to 110.13 U mL^{-1} (C/N molar ratio: 50.08). An increase in the biomass concentration of 41.81 g L^{-1} over the level of batch fermentation, to 74.85 g L^{-1}, was obtained, with a reduction in the C/N molar ratio. In addition, a high xylanase activity of 101.3 U mL^{-1} was obtained at a C/N molar ratio of 21.91.

However, the cell density and xylanase activity both declined when the C/N ratio was further reduced (C/N 15.93 and 12.52). This result suggests that a lower C/N molar ratio could inhibit cell growth and recombinant protein synthesis, particularly when a higher yeast extract concentration was fed to the culture system. The presence of excess nitrogen (30–40 g L^{-1}) may lead to yeast cell death and potentially to a stuck fermentation process. This phenomenon is especially common in the wine-making process, in which an excess of nitrogen can trigger cell death in certain situations [35]. Additionally, other authors have found [36] that cellular salt homoeostasis in *K. lactis* is strongly dependent on mitochondrial respiration and/or on the ion homoeostasis of the mitochondria themselves, which could be a primary target of salt stress. In our case, the salt content increased proportionally with the increase in the yeast extract supplement. Therefore, only a low nitrogen concentration was required to maintain the sustenance of the viable cell.

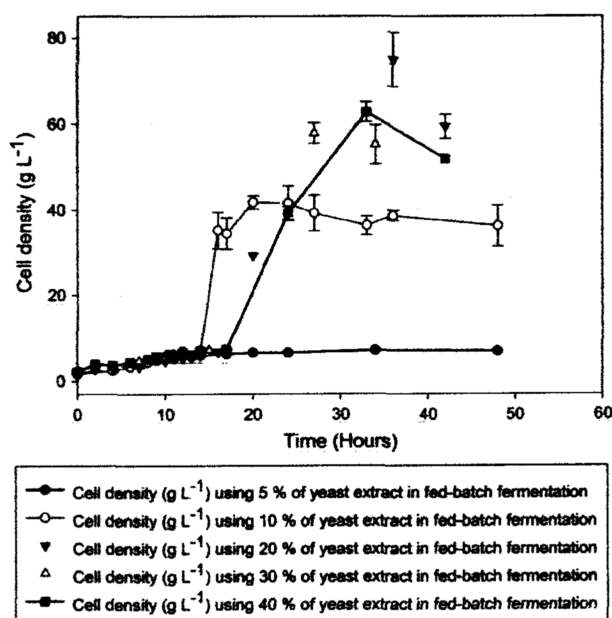

Fig. 4 Effects of various yeast extract concentrations on the biomass production (5, 10, 20, 30 and 40 g L^{-1}) of fed-batch fermentation systems

Table 4 Effect of yeast extract concentrations in the feeding medium on the recombinant xylanase and biomass production

Properties	Batch	Fed-batch						
		1.5-L					5-L	
Initial glucose concentration (g L^{-1})	20	200	200	200	200	200	400	500
Initial yeast extract concentration (g L^{-1})	–	5	10	20	30	40	20	20
C/N molar ratio	8.76	50.08	35.05	21.91	15.93	12.52	43.82	54.77
Maximum recombinant xylanase activity (U mL^{-1})	75.53	110.13	63.07	101.30	89.52	97.14	107.12	465.07
Time to reach maximum recombinant xylanase production (h)	6	20	10	16	11	17	13	18
Maximum biomass concentration (g L^{-1})	7.56	7.12	41.81	74.85	57.84	62.75	56.42	44.60
Time to reach maximum biomass production (h)	24	36	20	36	27	32	35	24
Maximum ethanol produced (g L^{-1})	0.03	10.77	10.78	6.27	11.00	10.30	–	–
Residual glucose (g L^{-1})	0.04	0.07[a]	0.19[a]	0.07[a]	2.66[a]	0.40[a]	–	–
Improvement of recombinant xylanase activity compared to the batch (%)		45.6	−16.5	34.2	18.5	28.7	41.9	515.7
Final pH	4.3	5.1	5.2	4.9	5.0	5.3	–	–

[a] Residual glucose after 48 h of fermentation

Table 5 Comparison of the feeding strategies for the production of enzymes by recombinant *K. lactis*

Host	Protein/enzyme	Feeding strategy	Volume culture (L)	Maximum yield		References
				Cell density (g L^{-1})	Protein/enzyme activity	
K. lactis GG799	Xylanase	Exponential feeding; C/N ratio feed	1.5	74.8	101.3 U mL^{-1}	This study
K. lactis GG799	Xylanase	Exponential feeding; C/N ratio feed	5	44.6	465.07 U mL^{-1}	This study
K. lactis JA6	Glucoamylase	Exponential and constant feeding; lactose feed	2	25	17 U mL^{-1}	[13]
K. lactis K7	Human lysozyme	Constant feeding; lactose feed and pH control	1.25	4.9	36.1 U mL^{-1}	[11]
K. lactis sp.	Bovine pancreatic trypsin inhibitor	Exponential feeding; glucose/galactose ratio feed	7	26	0.9 mg L^{-1}	[12]

However, Hahn-Hagerdal et al. [37] stated that complex additives have been shown to reduce product proteolysis. Yet, rather than actually reducing the proteolysis, complex nutrients may compensate for the process by providing large amounts of key compounds for the prolonged maintenance of efficient protein synthesis. Therefore, to determine whether xylanase can be overexpressed, especially in high cell density cultures, the initial yeast extract concentration was fixed at 20 g L^{-1}, while the glucose concentration was increased to 400 and 500 g L^{-1}. Surprisingly, the xylanase expression rates under glucose feeding concentrations of 400 and 500 g L^{-1} were 107.12 and 465.07 U mL^{-1}, respectively.

Interestingly, a C/N molar ratio of 54.77 (under a 500 g L^{-1} glucose feeding concentration) substantially increased the production of xylanase, up to 6.2-fold compared to the batch cultures. Changes in the C/N ratio due to higher glucose concentrations were found to significantly improve the xylanase activity compared with the excess nitrogen. One possible explanation for this observation is that the excess glucose prevents any glucose insufficiency to act as inducer of xylanase expression because, as previously stated, glucose is mainly used by or directed to the central metabolic pathways of energy generation. In addition, because *K. lactis* is a respiro-fermentative yeast, it is hypothesised that increasing the DO level would be ideal, as this could lead to an increase in the xylanase production during the same period of time. This finding, together with the success of the controlled strategy, indicated that a one-step exponential feeding strategy is the best operational strategy for the overexpression of recombinant protein production. The result contradicts the claim by Ruiz et al. [38] that a constant feeding profile after induction has a favourable effect on the amount of recombinant protein produced in *E. coli*. Table 5 summarises other studies that have discussed feeding strategies as a means to optimise the process using fed-batch cultivation.

Conclusion

This study demonstrates the success of the exponential feeding strategy for recombinant xylanase production during fed-batch fermentation. A low level of yeast extract was sufficient to produce high protein production levels. In addition, the ratio of the glucose concentration to the nitrogen concentration affected the quality of the protein produced. In the present study, a C/N ratio of 54.77 led to the highest xylanase activity (up to 6.2-fold), which was produced using high cell density cultures of *K. lactis* GG799.

Acknowledgements This project was supported by the Genomics and Molecular Biology Initiatives Programme of the Malaysia Genome Institute, Ministry of Science, Technology and Innovation Malaysia (Project No. 07-05-MGI-GMB012). And to late Dr Amir Rabu who has passed away seven months ago, farewell Dr, we will continue the walk you have started.

References

1. Baumann K, Carnicer M, Dragosits M, Graf AB, Stadlmann J, Jouhten P, Maaheimo H, Gasser B, Albiol J, Mattanovich D, Ferrer P (2010) A multi-level study of recombinant *Pichia pastoris* in different oxygen conditions. BMC Syst Biol 4:141. doi:10.1186/1752-0509-4-141
2. Breunig KD, Bolotin-Fukuhara M, Bianchi MM, Bourgarel D, Falcone C, Ferrero II, Frontali L, Goffrini P, Krijger JJ, Mazzoni C, Milkowski C, Steensma HY, Wesolowski-Louvel M, Zeeman AM (2000) Regulation of primary carbon metabolism in *Kluyveromyces lactis*. Enzym Microb Technol 26(9–10):771–780. doi:10.1016/S0141-0229(00)00170-8
3. Van Ooyen AJ, Dekker P, Huang M, Olsthoorn MM, Jacobs DI, Colussi PA, Taron CH (2006) Heterologous protein production in the yeast *Kluyveromyces lactis*. FEMS Yeast Res 6(3):381–392. doi:10.1111/j.1567-1364.2006.00049.x
4. Merico A, Capitanio D, Vigentini I, Ranzi BM, Compagno C (2004) How physiological and cultural conditions influence heterologous protein production in *Kluyveromyces lactis*. J Biotechnol 109(1–2):139–146. doi:10.1016/j.jbiotec.2003.10.031
5. Glick BR (1995) Metabolic load and heterologous gene expression. Biotechnol Adv 13(2):247–261. doi:10.1016/0734-9750(95)00004-A
6. Trentmann O, Khatri NK, Hoffmann F (2004) Reduced oxygen supply increases process stability and product yield with recombinant *Pichia pastoris*. Biotechnol Prog 20(6):1766–1775. doi:10.1021/bp049711h
7. Backhaus K, Heilmann CJ, Sorgo AG, Purschke G, de Koster CG, Klis FM, Heinisch JJ (2010) A systematic study of the cell wall composition of *Kluyveromyces lactis*. Yeast 27(8):647–660. doi:10.1002/yea.1781
8. Hsieh HB, Da Silva NA (2000) Development of a LAC4 promoter-based gratuitous induction system in *Kluyveromyces lactis*. Biotechnol Bioeng 67(4):408–416. doi:10.1002/(sici)1097-0290(20000220)67:4<408:aid-bit4>3.0.co;2-0
9. Zhang J, Reddy J, Buckland B, Greasham R (2003) Toward consistent and productive complex media for industrial fermentations: studies on yeast extract for a recombinant yeast fermentation process. Biotechnol Bioeng 82:640–652
10. Srinivasan S, Barnard GC, Gerngross TU (2002) A novel high-cell-density protein expression system based on *Ralstonia eutropha*. Appl Environ Microbiol 68(12):5925–5932. doi:10.1128/aem.68.12.5925-5932.2002
11. Huang EL, Demirci A (2009) Enhanced human lysozyme production by *Kluyveromyces lactis*. Food Bioprocess Technol 2(2):222–228. doi:10.1007/s11947-008-0062-1
12. Panuwatsuk W, Da Silva NA (2003) Application of a gratuitous induction system in *Kluyveromyces lactis* for the expression of intracellular and secreted proteins during fed-batch culture. Biotechnol Bioeng 81(6):712–718. doi:10.1002/bit.10518
13. Paciello L, Romano F, Alteriis E, Parascandola P, Romano V (2010) Glucoamylase by recombinant *Kluyveromyces lactis* cells: production and modelling of a fed batch bioreactor. Bioprocess Biosyst Eng 33(4):525–532. doi:10.1007/s00449-009-0364-3
14. Fuzi S, Mahadi N, Jahim J, Murad A, Bakar F, Jusoh M, Rahman R, Illias R (2012) Development and validation of a medium for recombinant endo-β-1,4-xylanase production by *Kluyveromyces lactis* using a statistical experimental design. Ann Microbiol 62(1):283–292. doi:10.1007/s13213-011-0258-x
15. Athmaram TN, Singh A, Saraswat S, Srivastava S, Misra P, Kameswara Rao M, Gopalan N, Rao PVL (2013) A simple *Pichia pastoris* fermentation and downstream processing strategy for making recombinant pandemic swine origin influenza A virus hemagglutinin protein. J Ind Microbiol Biotechnol 40(2):245–255. doi:10.1007/s10295-012-1220-z
16. Cregg J, Cereghino J, Shi J, Higgins D (2000) Recombinant protein expression in *Pichia pastoris*. Mol Biotechnol 16(1):23–52. doi:10.1385/mb:16:1:23
17. Cunha AE, Clemente JJ, Gomes R, Pinto F, Thomaz M, Miranda S, Pinto R, Moosmayer D, Donner P, Carrondo MJ (2004) Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*. Biotechnol Bioeng 86(4):458–467. doi:10.1002/bit.20051
18. Wang X, Xu P, Yuan Y, Liu C, Zhang D, Yang Z, Yang C, Ma C (2006) Modeling for Gellan Gum production by *Sphingomonas paucimobilis* ATCC 31461 in a simplified medium. Appl Environ Microbiol 72(5):3367–3374. doi:10.1128/aem.72.5.3367-3374.2006
19. Bailey MJ, Biely P, Poutanen K (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23(3):257–270. doi:10.1016/0168-1656(92)90074-J
20. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428. doi:10.1021/ac60147a030
21. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3
22. Luedeking R, Piret EL (2000) A kinetic study of the lactic acid fermentation. Batch process at controlled pH. Biotechnol Bioeng 67(6):636–644. doi:10.1002/(sici)1097-0290(20000320)67:6<636:aid-bit3>3.0.co;2-u
23. Ghosalkar A, Sahai V, Srivastava A (2008) Optimization of chemically defined medium for recombinant *Pichia pastoris* for biomass production. Bioresour Technol 99(16):7906–7910. doi:10.1016/j.biortech.2008.01.059
24. Boender LGM, de Hulster EAF, van Maris AJA, Daran-Lapujade PAS, Pronk JT (2009) Quantitative physiology of *Saccharomyces cerevisiae* at near-zero specific growth rates. Appl Environ Microbiol 75(17):5607–5614. doi:10.1128/aem.00429-09
25. Sawale S, Lele SS (2010) Statistical optimization of media for dextran production by *Leuconostoc* sp., isolated from fermented idli batter. Food Sci Biotechnol 19(2):471–478. doi:10.1007/s10068-010-0066-2

26. Tappe W, Laverman A, Bohland M, Braster M, Rittershaus S, Groeneweg J, Van Verseveld HW (1999) Maintenance energy demand and starvation recovery dynamics of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* cultivated in a retentostat with complete biomass retention. Appl Environ Microbiol 65:2471–2477
27. Møller K, Christensen B, Förster J, Piškur J, Nielsen J, Olsson L (2002) Aerobic glucose metabolism of *Saccharomyces kluyveri*: growth, metabolite production, and quantification of metabolic fluxes. Biotechnol Bioeng 77(2):186–193. doi:10.1002/bit.10122
28. Møller K, Olsson L, Piškur J (2001) Ability for anaerobic growth is not sufficient for development of the petite phenotype in *Saccharomyces kluyveri*. J Bacteriol 183(8):2485–2489. doi:10.1128/jb.183.8.2485-2489.2001
29. Merico A, Galafassi S, Piškur J, Compagno C (2009) The oxygen level determines the fermentation pattern in *Kluyveromyces lactis*. FEMS Yeast Res 9(5):749–756. doi:10.1111/j.1567-1364.2009.00528.x
30. Manzoni M, Rollini MS, Benedetti A (2010) Copper-enriched biomass, method for the preparation thereof and pro-biotic, cosmetic, dietary and nutraceutic products comprising the same. Google Patents
31. Kershaw CJ, Brown NL, Constantinidou C, Patel MD, Hobman JL (2005) The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. Microbiology 151(4):1187–1198. doi:10.1099/mic.0.27650-0
32. Yi X, Shi Y, Xu H, Li W, Xie J, Yu R, Zhu J, Cao Y, Qiao D (2010) Hyperexpression of two *Aspergillus niger* xylanase genes in *Escherichia coli* and characterization of the gene products. Braz J Microbiol 41(3):778–786
33. Ponzo JH, Weigand WA (1991) Simple structured model for α-amylase synthesis by *Bacillus amyloliquefaciens*. Biotechnol Bioeng 38(9):1065–1081. doi:10.1002/bit.260380916
34. Görgens J, Zyl W, Knoetze J, Hahn-Hägerdal B (2005) Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium. Appl Microbiol Biotechnol 67(5):684–691. doi:10.1007/s00253-004-1803-3
35. Tesnière C, Delobel P, Pradal M, Blondin B (2013) Impact of nutrient imbalance on wine alcoholic fermentations: nitrogen excess enhances yeast cell death in lipid-limited must. PLoS One 8(4):e61645. doi:10.1371/journal.pone.0061645
36. Goffrini P (2007) A respiratory-deficient mutation associated with high salt sensitivity in *Kluyveromyces lactis*. FEMS Yeast Res 7(2):180–187. doi:10.1111/j.1567-1364.2006.00148.x
37. Hahn-Hagerdal B, Karhumaa K, Larsson C, Gorwa-Grauslund M, Gorgens J, van Zyl W (2005) Role of cultivation media in the development of yeast strains for large scale industrial use. Microb Cell Fact 4(1):31. doi:10.1186/1475-2859-4-31
38. Ruiz J, Fernández-Castané A, Mas C, González G, López-Santín J (2013) From laboratory to pilot plant *E. coli* fed-batch cultures: optimizing the cellular environment for protein maximization. J Ind Microbiol Biotechnol 40(3–4):335–343. doi:10.1007/s10295-012-1226-6