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ORIGINAL ARTICLE

Development and validation of a medium for recombinant endo-β-1,4-xylanase production by *Kluyveromyces lactis* using a statistical experimental design

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Abstract *Kluyveromyces lactis* is an excellent host for a high cell density culture, which allows high expression levels of recombinant enzymes. Nutrient composition and culture conditions affect the secretion, production level and stability of the recombinant host. Therefore, it is technologically important to formulate a medium that stimulates high cell density and enhances the desired enzyme production using *K. lactis* GG799. In this study, six media were initially compared, and a Plackett-Burman experimental design was employed to screen for important components and trace elements. Nitrogen sources such as ammonium sulfate and free amino acid (casamino acid) as well as compounds like $MgSO_4 \cdot 7H_2O$, Na_2SO_4, $ZnSO_4 \cdot 6H_2O$, $MnSO_4 \cdot 4H_2O$ and KH_2PO_4 affected biomass concentrations (5.67 g/l) and recombinant endo-β-1,4-xylanase (Xyn2) production (49.73 U/ml). Optimum productivity was obtained at shorter incubation times (i.e., 6 h), making the medium suitable for use when seeking efficient production. Expression of recombinant Xyn2 by *K. lactis* GG799 in the designed medium resulted in satisfactory recombinant Xyn2 volumetric productivity (v_p) at 8.29 U/ml/h. When compared to the rich, non-selective YPD medium, the designed medium improved biomass output and recombinant Xyn2 production in *K. lactis* GG799 by approximately 9 and 22%, respectively.

Keywords Recombinant xylanase · Kluyveromyces lactis GG799 · Plackett-Burman · Medium development

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Introduction

Xylanases (EC 3.2.1.8) play important roles in industrial processes, where they are used as additives to improve the quality of baked goods (Collins et al. 2006; Basinskiene et al. 2008) and animal feeds and to bleach kraft pulp. Xylanases also act synergistically with other hemicellulases to produce commercial xylooligosaccharides (Jiang et al. 2004). Most commercial xylanase preparations originate from microorganisms belonging to the genus *Trichoderma* and *Aspergillus* (Ahmed et al. 2009). However, extensive purification to isolate the pure enzyme from fungi is

necessary, especially in large-scale production (Bauer et al. 2006). Therefore, cloning genes into a recombinant system is a prerequisite to obtain reproducible amounts of desired enzyme with specific properties (Bleve et al. 2008). One of the most promising hosts for gene cloning is *Kluyveromyces lactis* (Van Ooyen et al. 2006; Wamalwa et al. 2007). *Kluyveromyces lactis* is a unicellular organism that is phylogenetically close to *Saccharomyces cerevisiae,* as shown in comparison studies (Pain et al. 2004) subsequent to the whole genome sequencing of *K. lactis* (Sherman et al. 2004). The sequencing effort led to important breakthroughs in utilizing *K. lactis* specifically in biotechnological and industrial processes, as well as in regulatory network evolutions and their functional interactions (Bussereau et al. 2006; Rodicio et al. 2006). In addition, most *K. lactis* strains require a methanol-free media for growth. This requirement represents a major production advantage because explosion-proof fermentation equipment and high cost carbon sources are not needed, unlike in the large-scale growth of some methylotrophic yeasts (Mayer et al. 1999; Van Ooyen et al. 2006).

Despite the importance of the genetic background, the composition of cultivation media is crucial to the successful development of strains for large-scale industrial production of heterologous proteins (Hahn-Hagerdal et al. 2005). A cultivation medium is designed to reflect the biosynthetic capacity of microorganisms, which consequently affects strain analyses and performance in industrial applications. Chemically defined media are usually preferred in laboratory research because they permit one to determine specific requirements for growth and product formation (Hensing et al. 1995).

The classical procedure for optimizing fermentation media is by conducting one-dimensional searches with successive variable variations [or the one factor at a time (OFAT) method]. However, it is practically impossible for OFAT methods to achieve an optimal medium in a finite number of experiments (Ibrahim and Elkhidir 2011). Therefore, statistical designs are used to reduce experiment numbers despite large variable numbers. Statistical experimental designs are also important in distinguishing variables with significant effects on the desired response. For example, key variables can be revealed by employing fractional factorial designs, such as the Plackett-Burman method (Plackett and Burman 1946) and two-level factorial approach (Lundstedt et al. 1998). The Plackett-Burman statistical design is preferable to classical methods because it is rapid and reliable, highlights important nutrients and substantially reduces the number of experiments required, saving time, chemicals and manpower (Srinivas et al. 1994; Murthy et al. 2000).

Several papers have been published on the development and optimization of media for the production of recombinant proteins in hosts such as *Pichia pastoris* (Chang et al. 2006), *S. cerevisiae* (Zhang et al. 2007; Larsson et al. 2001) and *Escherichia coli* strains (Beshay et al. 2003). However, optimal medium conditions have not been developed and published for *K. lactis.* Therefore, this study was undertaken to develop a medium for the high expression level of a *Trichoderma reesei* Xyn2 in *K. lactis* GG799 strain (a wild-type industrial isolate from DSM Food Specialties, Delft, Netherlands) using a statistical experimental approach. The results presented below should provide a firm basis for developing an industrial process of recombinant enzyme production in *K. lactis* GG799.

Materials and methods

Recombinant strain and construction of expression vector

Kluyveromyces lactis GG799 strain (see *K. lactis* Protein Expression Kit Instruction Manual; New England Biolabs, USA) was used as a host for the expression of recombinant Xyn2 from *T. reesei* ATCC 58350. Prior to cloning the gene, reverse transcription polymerase chain reaction (RT-PCR) amplifications were carried out using primers designed from an mRNA sequence of *T. reesei* strain QM6a (NCBI accession number: U24191). Oligonucleotide primers were designed as follow: XynF 5′ AATGTCGACC AGACGATTCAGCCCGGCACGGGCTACAAC-3′ and XynR 5′-AATGCGGCCGCTTTAGCTTAGCTGACGGT GATGGAAGCAGA-3′ supplemented with *SalI* and *NotI* restriction sites, respectively. The endo-β-1,4-xylanase gene (*xyn2*) was cloned into vector pKLAC1 (9,091 bp) located downstream of the *K. lactis* α-mating factor (α_{MF}) signal sequence. The vector is based on the $P_{LAC\text{-}PBI}$ variant that has mutations in the Pribnow box-like sequence (PBI) containing the strong *LAC4* promoter (P_{LAC4}) for expression of endoxylanase. The vector also contained the Xyn2 and the *ADH2* promoter (P_{ADH2}), which drives expression of an acetamidase selectable marker gene (*amdS*). The constructed pKLAC1*xyn2* vector was linearized with *SacII* before being integrated into the *K. lactis* genome at the *LAC4* promoter locus. Transformant selection was carried out by growing recombinant cultures harboring pKLAC1-*xyn2* on a nitrogen-free minimal medium with a yeast carbon base (YCB) containing 5 mM acetamide.

Growth medium and conditions

Recombinant *K. lactis* GG799 was routinely cultured in 100 ml of yeast extract peptone dextrose (YPD) medium containing 20 g/l of glucose as a carbon source (Table 1); the medium was buffered to pH 5.0±0.2 using a 50 mM phosphate-citrate buffer. Media and glucose preparations were autoclaved separately at 121°C for 15 min. The same

Table 1 Medium composition for preliminary screening phase. All media were supplied with 20 g/l glucose as carbon source

Medium and reference	Compositions (in 1 l)
Van Hoek medium labeled as YHP (Van Hoek et al. 2000)	$(NH_4)_2SO_4$, 15 g; KH_2PO_4, 8.0 g; $MgSO_4$, 3.0 g; $ZnSO_4$, 0.4 g
Semi-synthetic medium labeled as SS (Domingues et al. 2005)	KH_2PO_4, 5 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4{\cdot}7H_2O$, 0.4 g; yeast extract, 2.0 g
Modified Knoll medium labeled as CSc (Knoll et al. 2007)	KH_2PO_4, 5.55 g; $(NH_4)_2SO_4$, 7 g; $MgSO_4{\cdot}7H_2O$, 2.76 g; Na_2SO_4, 0.34 g; $CaCl_2{\cdot}H_2O$, 2.63 g; $MnSO_4{\cdot}H_2O$, 0.035 g; $ZnSO_4{\cdot}7H_2O$, 0.0263 g; $CuSO_4{\cdot}5H_2O$, 0.007 g
PCT medium (Srinivasan et al. 2002)	$MgSO_4{\cdot}7H_2O$, 2.2 g; K_2SO_4, 3.0 g; Na_2SO_4, 0.18 g; $FeSO_4{\cdot}7H_2O$, 0.18 g; $CuSO_4{\cdot}5H_2O$, 0.0005 g; $ZnSO_4{\cdot}6H_2O$, 0.0024 g; $MnSO_4{\cdot}4H_2O$, 0.0024 g; $CaCl_2{\cdot}2H_2O$, 0.0624 g
Biostat medium labeled as M5 (Sartorius Stedim, Germany 2009)	$(NH_4)_2SO_4$, 2.0 g; $K_2HPO_4{\cdot}3H_2O$, 2.0 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; KCI 2.0 g; yeast extract, 0.1 g
YPD (New England Biolabs, USA)	Yeast extract, 10 g; peptone from meat, 20 g

initial glucose concentration and pH conditions were used throughout the study. All chemicals were purchased from Sigma-Aldrich (USA) and Merck (Germany) unless otherwise stated. Cells were incubated at 30±0.5°C with shaking at 250 rpm until growth reached the mid-exponential phase (i.e., A_{660} of 17). Thereafter, cultures were centrifuged at 6,000 rpm for 10 min and washed twice with sterile distilled water. Cultures were resuspended and concentrated to 20–30 ml prior to use in inoculations.

Preliminary screening

A preliminary screening experiment was carried out to verify mineral components needed for growth and secretion of the recombinant Xyn2. A 10% *K. lactis* GG799 inoculum, having an A_{660} of 5–6 (unless otherwise mentioned), was cultivated under sterilized conditions in 50 ml of media as shown in Table 1. Cultures were incubated at 30±0.5°C with shaking at 250 rpm. Variable components in the Plackett-Burman design were chosen based on results of biomass and xylanase activity. Software by Design Expert (Statistics Made Easy, v. 6.0.4, Stat-Ease, Minneapolis, MN, USA, 2001) was used to generate the experimental design.

Screening of nutrient components using the Plackett-Burman method

Eleven independent variables in twelve combinations were organized according to the Plackett-Burman design matrix (Table 2). Variables and concentrations were obtained from the preliminary experiment performed. Inoculum with a final A_{660} of 10 was seeded in 250 ml flasks containing 50 ml of media and incubated at 30°C±0.5°C on a rotary shaker at 250 rpm. Samples of 3 ml were collected and centrifuged at 6,000 rpm for 10 min, and the resulting supernatant was used for the xylanase assay.

Experimental design and statistical analysis

A Plackett-Burman experimental design was used to determine the most important media components for recombinant Xyn2 expression by *K. lactis* GG799. Design Expert software was used to fit the first-order model of a linear approach with $Y(x,\beta) = \beta_o + \sum_{i=1}^{n} \beta_i x_i$ where Y(x,β) is the estimated response (i.e., xylanase produced), β_o and β_i are constant regression coefficients of the model, and x_i represents independent variables (i.e., media components). Results of a regression analysis (ANOVA) were used to determine significance levels and screen out non-significant media components.

Determination of biomass concentration

The biomass concentration was determined by turbidity measurements at an absorbance of 660 nm using a UV/visible spectrophotometer (Ultrospec 1100 Pro; Amersham Bioscience, Sweden). Measured values were correlated to dry weight from duplicate samples (2 ml taken every 2 h during cultivation) that were centrifuged at 6,000 rpm for 10 min, washed twice with distilled water and dried for at least 24 h at 80°C. The equation to calculate dry mass was as follows:

$$\text{dry mass}(g/l) = 0.379 \times A_{660}.$$

Xylanase assay

Xylanase activity was measured using a dinitrosalicylic acid method (Miller 1959) with 0.01 g/l of beechwood xylan (Sigma) as the substrate at 50°C. Appropriate dilutions 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

Table 2 Plackett-Burman experimental design combinations of factors and levels for recombinant Xyn2 and biomass production using *K. lactis* GG799

Run no.	Levels	Variables											Responses	
		$(NH_4)_2SO_4$[a] X_1 (g/l)	$MgSO{\cdot}7H_2O$[a] X_2 (g/l)	Na_2SO_4[b] X_3 (g/l)	$FeSO_4{\cdot}7H_2O$[a] X_4 (g/l)	Casamino acid[c] X_5 (g/l)	$CuSO_4{\cdot}5H_2O$[b] X_6 (g/l)	$ZnSO_4{\cdot}6H_2O$[a] X_7 (g/l)	$MnSO{\cdot}4H_2O$[b] X_8 (g/l)	$CaCl{\cdot}2H_2O$[b] X_9 (g/l)	K_2SO_4[b] X_{10} (g/l)	KH_2PO_4[a] X_{11} (g/l)	Xylanase activity (U/ml)	Biomass concentration (g/l)
	+	15.00	3.00	0.18	0.003	5.00	0.005	0.40	0.005	0.003	3.00	8.00		
	−	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
1		−	+	−	−	−	+	+	+	−	+	+	40.73	4.99
2		−	−	−	−	−	−	−	−	−	−	−	40.81	4.52
3		−	−	+	+	+	−	+	+	−	+	−	39.57	4.94
4		+	−	+	+	−	+	−	−	−	+	+	45.56	4.08
5		−	−	−	+	+	+	−	+	+	−	+	40.96	5.38
6		+	+	−	+	−	−	−	+	+	+	−	19.52	5.34
7		+	+	−	+	+	−	+	−	−	−	+	19.49	5.53
8		+	+	+	−	+	+	−	+	−	−	−	36.92	5.24
9		−	+	+	+	−	+	+	−	+	−	−	43.26	4.55
10		−	+	+	−	+	−	−	−	+	+	+	20.71	5.85
11		+	−	+	−	−	−	+	+	+	−	+	41.91	4.60
12		+	−	−	−	+	+	+	−	+	+	−	23.57	5.44

Each row represents an experiment, and each column represents an independent variable, two level variations and responses. Positive and negative signs represent different levels (higher and lower) of the variable examined. Responses include the activity of recombinant xylanase and biomass production from the screening stage using a Plackett-Burman experimental design

The concentration of the components was obtained from the preliminary screening phase ([a] Van Hoek et al. 2000, [b] Srinivasan et al. 2002), while the concentration for casamino acid was obtained from [c] Panuwatsuk and Da Silva (2003)

sugars. One unit of xylanase was defined as the amount of enzyme required to liberate one μmole of xylose from xylan per minute under assay conditions.

Validation of the Plackett-Burman experimental model

The mathematical model generated from Plackett-Burman implementation was validated by conducting an experiment with a predicted medium set for maximum responses. The experiment was performed in the same manner as the screening phase.

Results and discussion

Expression of recombinant Xyn2 in *K. lactis* GG799

A cDNA fragment encoding *T. reesei* Xyn2 and 191 amino acid residues was obtained by RT-PCR (data not shown). The amino acid sequence is identical to *T. reesei* strain QM6a *xyn2* cDNA sequence (GenBank accession number U24191). The integrative plasmid pKLAC1*xyn2* produced polypeptides consisting of an N-terminal signal peptide of the *K. lactis* α_{MF} and the family 11 xylanase. Transformation of *K. lactis* GG799 was achieved by a chemical method that used transformation reagents supplied with the NEB yeast transformation kit. Colonies of recombinant *K. lactis* GG799 cells with the pKLAC1*xyn2* construct containing the *amdS* gene were isolated on yeast carbon base (YCB agar medium that lacked a nitrogen source but contained acetamide). Acetamidase expression in transformed cells allows for cell growth because acetamidase metabolizes acetamide to ammonia, which can be utilized by cells as a nitrogen source. The recombinant strain was then grown in YPD medium at 30°C in shaking flasks.

An advantage of *K. lactis* GG799 that makes this yeast especially attractive as a host system is that the recombinant plasmid has good stability in shake-flasks and pilot-scale cultures (Merico et al. 2004). As stated by Van Ooyen et al. (2006), a major benefit in using integrative vectors is the increased of genetic stability of the transformed strain compared to episomal vectors. Other than that, selection of transformant using acetamide influences the frequency of targeted tandem integration of a vector into the chromosome of *K. lactis* GG799 cells. According to Read et al. (2007), acetamide selection not only almost completely enriches populations for strains harboring multiple tandem vector integrations but also increases vector copy numbers compared to antibiotic selection. Transformation using expression vector pKLAC1 routinely yields strains bearing two to six integrated copies in more than 90% of the transformant on medium containing acetamide (Van Ooyen et al. 2006). In this study, whole-cell PCR strategies are used to detect targeted integration of pKLAC1*xyn2* in *K. lactis* GG799 chromosome (as recommended by the manufacturer). The PCR results exhibited multicopy integration in *K. lactis* genome (data not shown).

Preliminary experiment

It is essential to have extensive knowledge when formulating a medium so as to include important minerals and growth factors. However, not all nutrients enhance cell growth and protein production when present above a certain concentration (Lee 1996). To determine which basic components are vital for cell growth and production, six different media with different components and concentrations were tested before implementing the Plackett-Burman design. The effects of the media towards recombinant Xyn2 production are shown in Fig. 1. The highest recombinant Xyn2 activity of 48.10 U/ml was obtained from YHP medium with a biomass concentration of 4.23 g/l. In contrast, recombinant Xyn2 activity attained in the YPD medium was the lowest at 40.85 U/ml, but this medium yielded the highest biomass concentration at 5.19 g/l. In yeast extract-containing mediums, such as YPD, sugars and lactate serve as auxiliary carbon sources and strongly affect yeast cultivation rates (Hahn-Hagerdal et al. 2005). Variable selections for the Plackett-Burman design were based on these media that produced the highest levels of recombinant Xyn2, regardless of biomass concentrations. In addition, the free amino acid casamino acid (Amresco, Ohio, USA) was included in the process design because Jahic et al. (2006)

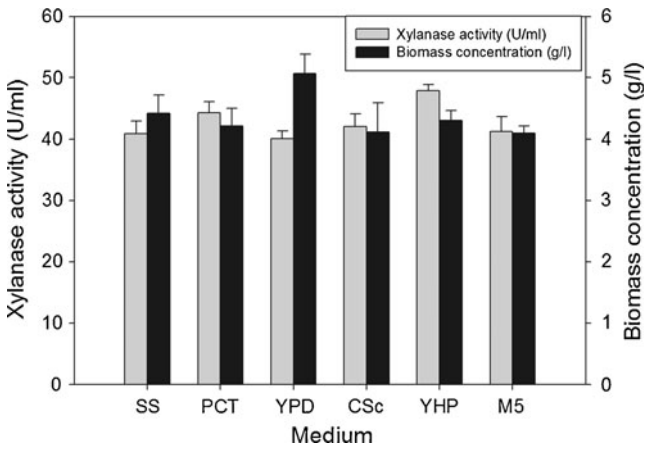

Fig. 1 Effects of six different media on recombinant Xyn2 production by *K. lactis* GG799 and biomass concentrations. The inoculum was cultured in 50 ml of each media with an A_{660} of 5 to 6 and maintained at 30°C with continuous shaking at 250 rpm (see "Materials and methods"). Each *bar* represents (▭) recombinant xylanase activity produced and (■) biomass concentrations in the media as the mean± standard error (n=3)

reported that it had a significant positive effect on growth and protein production in *P. pastoris*. Further medium improvement was carried out by combining casamino acid with components of YHP and PCT media because these two media have produced high expression of recombinant Xyn2 in *K. lactis* GG799, as shown in Fig. 1 and described in the initial screening process.

Statistical analysis by ANOVA

By using a Plackett-Burman experimental design (Table 2), wide variations in recombinant Xyn2 production were observed, ranging from 19.49±1.49 to 45.56±0.26 U/ml. These wide variations in recombinant Xyn2 activity were coupled with narrow variations in biomass that ranged from 4.08±0.18 to 5.85±0.03 g/l (Table 2). The wide variation in recombinant Xyn2 production reflected the significance of factors on the enzyme and biomass production (Purama and Goyal 2008) and vice versa. Comparisons among these eleven trials showed that the medium in trial 4 produced the highest recombinant Xyn2 activity, while the medium in trial 10 produced the highest biomass. However, influences of these media compositions were further analyzed using ANOVA.

Regression analyses data for recombinant Xyn2 and biomass production are shown in Tables 3 and 4, respectively. The model for recombinant Xyn2 production in *K. lactis* GG799 has a coefficient of determination (R^2)= 0.9976, meaning that 99.76% of the data variability can be elucidated effectively.

Each component was screened at the 95% confidence level regardless of its effects, either positive or negative. Among the 11 variables evaluated, only 8 factors, $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, Na_2SO_4, casamino acid, $CuSO_4{\cdot}5H_2O$, $CaCl_2{\cdot}2H_2O$, K_2SO_4 and $MnSO_4{\cdot}4H_2O$, had significant model terms based on the confidence level (Table 3). The remaining components studied including $FeSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}6H_2O$ and KH_2PO_4 were less than the 95% confidence level and therefore considered insignificant. In addition, apart from the percentage of the confidence level, main effects can be ranked based on the mean square. Table 3 demonstrates that casamino acid had the highest mean square value, supporting that it had a major impact on recombinant Xyn2 expression.

A positive coefficient intervals (CIs) (95% confidence level) show that the studied component will produce incremental responses if added and vice versa. If the influence of the component is equal to or greater than the 95% confidence level, and its coefficient values (CI low and CI high) are both negative, then the component is effective at enhancing recombinant Xyn2 production, but the concentration required is lower than the signified low (−) concentration (Gohel et al. 2006). However, if the CI low and CI high coefficient values are positive and negative, respectively, then the component has no effect on recombinant Xyn2 production. The medium components $CuSO_4{\cdot}5H_2O$, $MnSO_4{\cdot}4H_2O$ and Na_2SO_4 displayed positive coefficient values, suggesting that the concentrations of these three components should be increased to improve recombinant Xyn2 production. Conversely, casamino acid, $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$ and K_2SO_4 exhibited negative coefficients and thus should be reduced to improve recombinant Xyn2 production. Ignoring insignificant factors, the linear regression for recombinant Xyn2 production can be written as:

$$\text{Recombinant Xyn2 activity (U/ml)} = -3.26X_1 - 4.31X_2 + 3.57X_3 - 4.46X_5 + 4.33X_6 + 2.18X_8 - 2.51X_9 - 2.81X_{10}$$

Table 3 Analysis of ANOVA regression model for recombinant xylanase activity

Source	Sum of squares	Mean square	*F* value	Confidence level %	95% CI low	95% CI high
Model	1,195.11	149.39	156.88	99.92		
Casamino acid	238.97	238.97	250.94	99.95	−5.36	−3.57
$CuSO_4{\cdot}5H_2O$	225.07	225.07	236.35	99.94	3.43	5.23
$MgSO_4{\cdot}7H_2O$	223.17	223.17	234.36	99.94	−5.21	−3.42
Na_2SO_4	153.01	153.01	160.68	99.89	2.67	4.47
$(NH4)_2SO_4$	127.21	127.21	133.58	99.86	−4.15	−2.36
K_2SO_4	94.58	94.58	99.32	99.79	−3.70	−1.91
$CaCl_2{\cdot}2H_2O$	75.85	75.85	79.65	99.70	−3.41	−1.62
$MnSO_4{\cdot}4H_2O$	57.25	57.25	60.12	99.55	1.29	3.08
Residual	2.86	0.95				
Cor Total	197.97					

Table 4 Analysis of ANOVA regression model for biomass production

Source	Sum of squares	Mean square	*F* value	Confidence level %	95% CI low	95% CI high
Model	2.69	0.54	16.41	99.81		
Casamino acid	1.47	1.47	37.27	99.01	0.21	0.49
$MgSO_4{\cdot}7H_2O$	0.56	0.56	14.28	99.08	0.08	0.36
$CaCl_2{\cdot}2H_2O$	0.33	0.33	8.45	97.29	0.03	0.31
Na_2SO_4	0.27	0.27	6.85	96.02	−0.29	−0.01
Residual	0.2	0.033				
Cor total	2.89					

where

$X_1 = (NH_4)_2SO_4$; $X_2 = MgSO_4 \cdot 7H_2O$;

$X_3 = Na_2SO_4$; X_5= casamino acid;

$X_6 = CuSO_4 \cdot 5H_2O$; $X_8 = MnSO_4 \cdot 4H_2O$;

$X_9 = CaCl_2 \cdot 2H_2O$ and $X_{10} = K_2SO_4$.

For biomass production of *K. lactis* GG799, the model presented an R^2=0.9180, explaining 91.80% of the validity in the response. Similarly, components were screened at a 95% confidence level (Table 4). Among the 11 variables evaluated, only 4 (casamino acid, $MgSO_4{\cdot}7H_2O$, Na_2SO_4 and $CaCl_2{\cdot}2H_2O$) appeared to have significant model terms. The remaining components studied and not included in Table 4 [$(NH_4)_2SO_4$, $FeSO_4{\cdot}7H_2O$, K_2SO_4, $CuSO_4{\cdot}5H_2O$, $MnSO_4{\cdot}4H_2O$ and KH_2PO_4] had no significant effect on biomass production. Casamino acid (mean square of 1.47) appeared to be the most important factor in biomass production based on the relatively high mean square estimate. Specifically, casamino acid allowed for complete use of the carbon source and, consequently, enhanced cell growth and heterologous protein production (Merico et al. 2004). In addition, cultivation medium is composed of casamino acid, supplementing free amino acid requirements for heterologous protein production in recombinant yeast and relieving the metabolic burden of cells (Hahn-Hagerdal et al. 2005).

Na_2SO_4 was the only significant variable that had a negative coefficient value, while the other three variables (casamino acid, $MgSO_4{\cdot}7H_2O$ and $CaCl_2{\cdot}2H_2O$) showed positive coefficients. This outcome suggested that the Na_2SO_4 concentration should be decreased while concentrations of casamino acid, $MgSO_4{\cdot}7H_2O$ and $CaCl_2{\cdot}2H_2O$ should be increased to improve biomass production. The linear regression obtained for biomass production was:

$$\text{Biomass}(g/l) = 0.22X_2 - 0.15X_3 + 0.35X_5 + 0.17X_9$$

where

$X_2 = MgSO_4 \cdot 7H_2O$; $X_3 = Na_2SO_4$;

X_5 = casamino acid; $X_9 = CaCl_2 \cdot 2H_2O$

Validation of screening results

An experiment was performed to validate the efficiency of the Plackett-Burman-designed medium. Recombinant Xyn2 activity and biomass production were set at the maximum response level with the intention that the suggested medium would maximize both factors in *K. lactis* GG799. Highest predicted values of recombinant Xyn2 activity and biomass production were 45.8 U/ml and 5.25 g/l, respectively, in a medium containing 5 g/l $(NH_4)_2SO_4$, 1.69 g/l $MgSO_4{\cdot}7H_2O$, 0.18 g/l Na_2SO_4, 3.93 g/l casamino acid, 0.24 g/l $ZnSO_4{\cdot}6H_2O$, 1.0 g/l $MnSO_4{\cdot}4H_2O$ and 7.66 g/l KH_2PO_4. The recombinant Xyn2 activity attained was 49.73 U/ml, and biomass concentration was 5.67 g/l. Figure 2 represents the recombinant Xyn2 activity and biomass production profiles. Experimental and predicted values closely agreed with percent deviations of 7.9% for recombinant Xyn2 activity and 7.4% for biomass production. This outcome validated our findings for components screened under the Plackett-Burman design.

Expression of recombinant Xyn2 by *K. lactis* GG799 using the designed medium in a shake-flask culture provided satisfactory recombinant Xyn2 volumetric productivity (v_p). The maximum productivity for recombinant Xyn2 in *K. lactis* GG799 was 8.29 U/ml/h, which was eight-fold higher than productivity levels obtained by Wamalwa et al. (2007) from recombinant xylanase in *K. lactis* CBS 1065. The productivity level of recombinant Xyn2 from *T. reesei* produced by *K. lactis* was also seven-fold higher than levels obtained from *S. cerevisiae* (La Grange et al. 2001) and twice as high as production from the *P. pastoris* expression system (He et al. 2009). Moreover, the highest production of recombinant Xyn2

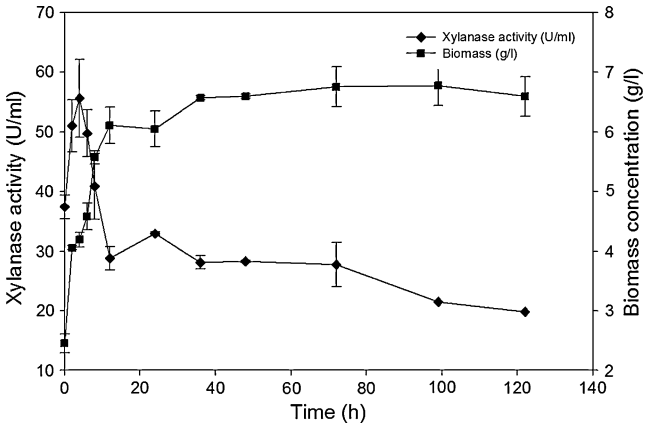

Fig. 2 Production of recombinant Xyn2 and cell growth of *K. lactis* GG799 profile. A validation experiment was performed to verify the efficiency of the optimal design medium in which both responses were set at their maximum level using statistical software. The method involves pulling together both first-order models and determining a set of conditions that is a compromise to the stated goals. Both (—◆—) production of recombinant xylanase and (—■—) cell growth of *K. lactis* GG799 were obtained using a designed medium after screening by Plackett-Burman methods. Each *line* represents the mean±standard error (n=3). The recombinant xylanase production started in the early exponential growth phase and increased to its maximum activity and later decreased when glucose concentrations became a limiting growth factor

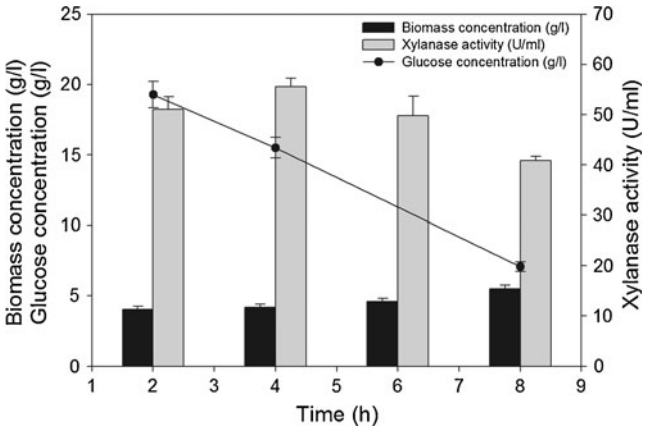

Fig. 3 The influence over time of glucose on recombinant Xyn2 production and biomass concentrations. An experiment was conducted to understand the relationship over time between glucose levels, recombinant xylanase expression and biomass production. The effects of (—●—) glucose concentration on (▭) recombinant xylanase and (■) biomass production were investigated. Samples of cultured *K. lactis* GG799 were taken after fermenting for at 2, 4 and 8 h of fermentation. Each *bar* and *line* represents the mean ± standard error

was reached within a relatively short time. Studies of xylanase production with *K. lactis* systems required more than 3 days using a complex medium (Faraco et al. 2008). Percent improvements in biomass concentration and recombinant Xyn2 production were approximately 9 and 22%, respectively, with use of the designed medium versus the YPD medium. Because the designed medium is superior to the YPD medium, we investigated the effects of different glucose concentration using the designed medium. Our findings revealed that higher glucose concentrations (5–50 g/l) resulted in increased cell biomass. However, at elevated glucose concentrations, the time required to reach maximum recombinant Xyn2 production was longer than at the original glucose concentration of 20 g/l glucose (data not shown).

An experiment was performed to understand relationships over the incubation time between glucose levels, recombinant xylanase expression and biomass production. Samples of cultured *K. lactis* GG799 were taken at 2, 4 and 8 h of fermentation during the validation phase. Supernatant from the centrifugation process was used to measure recombinant Xyn2 activity and glucose concentrations. The influence over time of glucose on recombinant Xyn2 production and biomass concentrations is shown in Fig. 3. Results show that Xyn2 activity decreased along with glucose concentration while biomass concentrations increased slightly. These data indicated that reductions of recombinant Xyn2 production in *K. lactis* GG799 may be linked to glucose depletions in the medium. As mentioned in "Materials and methods", the constructed vector pKLAC1*xyn2* was integrated at the *LAC4* locus of the genome. Glucose depletions can cause low Xyn2 expression levels because the *LAC4* expression system is mediated by glucose. Hsieh and Da Silva (2000) observed that glucose is an important inducer of recombinant enzyme production and also serves to provide carbon, energy and cell structure and cell maintenance. Thus, glucose depletions would reduce the synthesis of recombinant Xyn2 by *K. lactis* GG799. Conversely, feeding glucose into the designed medium of bioreactors may further increase recombinant Xyn2 production levels using *K. lactis* GG799.

The reduction in recombinant Xyn2 activity could also be due to proteolysis by proteases because *K. lactis* secretes proteases in the presence of glucose, galactose and glycerol (Madinger et al. 2009). Proteolytic degradation has been an ongoing concern for the production of recombinant proteins in other hosts (Zhou and Zhang 2002), such as *P. pastoris* (Sinha et al. 2005) and *S. cerevisiae* (Rao et al. 1999). On the other hand, a question arises on the stability of plasmid implicated in the production of Xyn2. In the present study, relatively, the integrated plasmid should be stable as showed by other *K. lactis* system published before. This is because chromosomal integration is a more stable option when compared to episomal maintenance of foreign DNA (Romanos et al. 1992). However, further studies are needed to confirm the integrated plasmid stability in expression of Xyn2 in *K. lactis* GG799.

Conclusion

Improved recombinant Xyn2 production in *K. lactis* was achieved using a statistical experimental design. These findings demonstrate that medium components play a significant role in the growth of *K. lactis* GG799 and recombinant Xyn2 production. Results of this study will facilitate the large-scale, economically viable production of xylanase using *K. lactis*. Moreover, enhanced recombinant Xyn2 productivity supports the use of the designed medium, especially because this enzyme is widely used and commercially important. Bioreactor studies are needed to further improve the production of recombinant Xyn2 in *K. lactis* GG799.

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References

Ahmed S, Riaz S, Jamil (2009) A Molecular cloning of fungal xylanases: an overview. Appl Microbiol Biotechnol 84:19–35. doi:10.1007/s00253-009-2079-4

Basinskiene L, Garmuviene S, Juodeikiene G (2008) Effects of enzymes and extruded wheat bran in fibre-enriched bread making. Foodbalt 15–19

Bauer S, Vasu P, Persson S, Mort AJ, Somerville CR (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. Proc Natl Acad Sci USA 103:11417–11422. doi:10.1073/pnas.0604632103

Beshay U, El-Enshasy H, Ismail IMK, Moawad H, Wojciechowska E, El-Ghany SA (2003) Beta-glucanase production from genetically modified recombinant *Escherichia coli*: Effect of growth substrates and development of a culture medium in shake flasks and stirred tank bioreactor. Process Biochem 39:307–313. doi:10.1016/S0032-9592(03)00078-5

Bleve G, Lezzi C, Mita G, Rampino P, Perrotta C, Villanova L, Grieco F (2008) Molecular cloning and heterologous expression of a laccase gene from *Pleurotus eryngii* in free and immobilized *Saccharomyces cerevisiae* cells. Appl Microbiol Biotechnol 79:731–741. doi:10.1007/s00253-008-1479-1

Bussereau F, Casaregola S, Lafay JF, Fukuhara MB (2006) The *Kluyveromyces lactis* repertoire of transcriptional regulators. FEMS Yeast Res 6:325–335. doi:10.1111/j.1567-1364.2006.00028.x

Chang SW, Shieh CJ, Lee GC, Akoh CC, Shaw JF (2006) Optimized growth kinetics of *Pichia pastoris* and recombinant *Candida rugosa* LIP1 production by RSM. J Mol Microbiol Biotechnol 11:28–40. doi:10.1159/000092817

Collins T, Hoyoux A, Dutron A, Georis J, Genot B, Dauvrin T, Arnaut F, Gerday C, Feller G (2006) Use of glycoside hydrolase family 8 xylanases in baking. J Cereal Sci 43:79–84. doi:10.1016/j.jcs.2005.08.002

Domingues L, Lima N, Teixeira JA (2005) *Aspergillus niger* beta-galactosidase production by yeast in a continuous high cell density reactor. Process Biochem 40:1151–1154. doi:10.1016/j.procbio.2004.04.016

Faraco V, Ercole C, Festa G, Giardina P, Piscitelli A, Sannia G (2008) Heterologous expression of heterodimeric laccase from *Pleurotus ostreatus* in *Kluyveromyces lactis*. Appl Microbiol Biotechnol 77:1329–1335. doi:10.1007/s00253-007-1265-5

Gohel V, Chaudhary T, Vyas P, Chhatpar HS (2006) Statistical screenings of medium components for the production of chitinase by the marine isolate *Pantoea dispersa*. Biochem Eng J 28:50–56. doi:10.1016/j.bej.2005.09.002

Hahn-Hagerdal B, Karhumaa K, Larsson CU, Gorwa-Grauslund M, Gorgens J, Van Zyl WH (2005) Role of cultivation media in the development of yeast strains for large scale industrial use. Microb Cell Fact 4:31. doi:10.1186/1475-2859-4-31

He J, Yu B, Zhang K, Ding X, Chen D (2009) Expression of endo-1, 4-beta-xylanase from *Trichoderma reesei* in *Pichia pastoris* and functional characterization of the produced enzyme. BMC Biotechnol 9:56. doi:10.1186/1472-6750-9-56

Hensing MCM, Bangma KA, Raamsdonk LM, de Hulster E, Van Dijken JP, Pronk JT (1995) Effects of cultivation conditions on the production of heterologous galactosidase by *Kluyveromyces lactis*. Appl Microbiol Biotechnol 43:58–64. doi:10.1007/BF00170623

Hsieh HB, Da Silva NA (2000) Development of a LAC4 promoter-based gratuitous induction system in *Kluyveromyces lactis*. Biotechnol Bioeng 67:408–416. doi:10.1002/(SICI)1097-0290(20000220)67:4<408::AID-BIT4>3.0.CO;2-0

Ibrahim HM, Elkhidir EE (2011) Response surface method as an efficient tool for medium optimisation. Trends Appl Sci Res 6:121–129. doi:10.3923/tasr.2011.121.129

Jahic M, Veide A, Charoenrat T, Teeri T, Enfors SO (2006) Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. Biotechnol Prog 22:1465–1473. doi:10.1021/bp060171t

Jiang ZQ, Deng W, Zhu YP, Li LT, Sheng YT, Hayashi K (2004) The recombinant xylanase B of *Thermotoga maritima* is highly xylan specific and produces exclusively xylobiose from xylans, a unique character for industrial applications. J Mol Catal B Enzym 27:207–213. doi:10.1016/j.molcatb.2003.11.012

Knoll A, Bartsch S, Husemann B, Engel P, Schroer K, Ribeiro B, Stockmann C, Seletzky J, Buchs J (2007) High cell density cultivation of recombinant yeasts and bacteria under non-pressurized and pressurized conditions in stirred tank bioreactors. J Biotechnol 132:167–179. doi:10.1016/j.jbiotec.2007.06.010

La Grange DC, Claeyssens IM, Pretorius IS, Van Zyl WH (2001) Degradation of xylan to D-xylose by recombinant *Saccharomyces cerevisiae* coexpressing the *Aspergillus niger* beta-xylosidase (*xlnD*) and the *Trichoderma reesei* xylanase II (*xyn2*) genes. Appl Environ Microbiol 67:5512–5519. doi:10.1128/AEM.67.12.5512-5519.2001

Larsson S, Cassland P, Jonsson LJ (2001) Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. Appl Environ Microbiol 67:1163–1170. doi:10.1128/AEM.67.3.1163-1170.2001

Lee SY (1996) High cell-density culture of *Escherichia coli*. Trends Biotechnol 14:98–105. doi:10.1016/0167-7799(96)80930-9

Lundstedt T, Seifert E, Abramo L et al (1998) Experimental design and optimization. Chemom Intell Lab Syst 42:3–40

Madinger CL, Sharma SS, Anton BP et al (2009) The effect of carbon source on the secretome of *Kluyveromyces lactis*. Proteomics 9:4744–4754. doi:10.1002/pmic.200800915

Mayer AF, Hellmuth K, Schlieker H et al (1999) An expression system matures: a highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*. Biotechnol Bioeng 63:373–381. doi:10.1002/(SICI)1097-0290(19990505)63:3<373::AID-BIT14>3.0.CO;2-T

Merico A, Capitanio D, Vigentini I, Ranzi BM, Compagno C (2004) How physiological and cultural conditions influence heterolo-

gous protein production in *Kluyveromyces lactis*. J Biotechnol 109:139–146. doi:10.1016/j.jbiotec.2003.10.031

Miller GL (1959) Use of dinitrosalicylic acid reagent for the determination of reducing sugar. Anal Chem 31:426–428

Murthy MSRC, Swaminathan T, Rakshit SK, Kosugi Y (2000) Statistical optimization of lipase catalyzed hydrolysis of methyl-oleate by response surface methodology. Bioprocess Biosyst Eng 22:35–39. doi:10.1007/PL00009097

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 81:712–718. doi:10.1002/bit.10518

Pain A, Bentley S, Parkhill J (2004) Genome watch: Eukaryotes: not beyond compare. Nat Rev Microbiol 2:856–857. doi:10.1038/nrmicro1028

Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 33:305–325. doi:10.1093/biomet/33.4.305

Purama RK, Goyal A (2008) Screening and optimization of nutritional factors for higher dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 using statistical approach. Bioresour Technol 99:7108–7114. doi:10.1016/j.biortech.2008.01.032

Rao KJ, Kim CH, Chung BH, Kim MK, Rhee SK (1999) Suppression of proteolytic degradation of recombinant hirudin from *Saccharomyces cerevisiae* using the O2-enriched air. Biotechnol Lett 21:391–394. doi:10.1023/A:1005443306866

Read JD, Colussi PA, Ganatra MB, Taron CH (2007) Acetamide selection of Kluyveromyces lactis cells transformed with an integrative vector leads to high-frequency formation of multicopy strains. Appl Environ Microbiol 73:5088–5096. doi:10.1128/AEM.02253-06

Rodicio R, Koch S, Schmitz HP, Heinisch JJ (2006) KlRHO1 and KlPKC1 are essential for cell integrity signalling in *Kluyveromyces lactis*. Microbiology 152:2635–2649. doi:10.1099/mic.0.29105-0

Romanos MA, Scorer CA, Clare JJ (1992) Foreign gene expression in yeast: a review. Yeast 8:423–488

Sartorius Stedim Biotech GmbH, Germany (2009) Biostat Aplus Recipes: *Saccharomyces cerevisiae* aerobic baker´s yeast fermentation, ver. 6. Publication No.: SBT1009-e09061

Sherman D, Durrens P, Beyne E, Nikolski M, Souciet JL (2004) Genolevures: comparative genomics and molecular evolution of hemiascomycetous yeasts. Nucleic Acids Res 32:D315–D318. doi:10.1093/nar/gkh091

Sinha J, Plantz BA, Inan M, Meagher MM (2005) Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: Case study with recombinant ovine interferon-T. Biotechnol Bioeng 89:102–112

Srinivas MRS, Naginchand, Lonsane BK (1994) Use of Plackett–Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the production of alpha-galactosidase by *Aspergillus niger* MRSS 234 in solid state fermentation. Bioprocess Eng 10:139–144. doi:10.1007/BF00369470

Srinivasan S, Barnard GC, Gerngross TU (2002) A novel high-cell-density protein expression system based on *Ralstonia eutropha*. Appl Environ Microbiol 68:5925–5932. doi:10.1128/AEM.68.12.5925-5932.2002

Van Hoek P, de Hulster E, Van Dijken JP, Pronk JT (2000) Fermentative capacity in high-cell-density fed-batch cultures of baker's yeast. Biotechnol Bioeng 68:517–523. doi:10.1002/(SICI)1097-0290(20000605)68:5<517::AID-BIT5>3.0.CO;2-O

Van Ooyen AJJ, Dekker P, Huang M, Olsthoorn MMA, Jacobs DI, Colussi PA, Taron CH (2006) Heterologous protein production in the yeast *Kluyveromyces lactis*. FEMS Yeast Res 6:381–392. doi:10.1111/j.1567-1364.2006.00049.x

Wamalwa BM, Zhao G, Sakka M, Shiundu PM, Kimura T, Sakka K (2007) High-level heterologous expression of *Bacillus halodurans* putative xylanase xyn11a (BH0899) in *Kluyveromyces lactis*. Biosci Biotechnol Biochem 71:688–693. doi:10.1271/bbb.60477

Zhang T, Wen S, Tan T (2007) Optimization of the medium for glutathione production in *Saccharomyces cerevisiae*. Process Biochem 42:454–458. doi:10.1016/j.procbio.2006.09.003

Zhou XS, Zhang YX (2002) Decrease of proteolytic degradation of recombinant hirudin produced by *Pichia pastoris* by controlling the specific growth rate. Biotechnol Lett 24:1449–1453. doi:10.1023/A:1019831406141