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Optimized Medium Improves Expression and Secretion of Extremely Thermostable Bacterial Xylanase, XynB, in *Kluyveromyces lactis*

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An extremely thermostable xylanase gene, *xynB*, from the hyperthermophilic bacterium *Thermotoga maritima* MSB8 was successful expressed in *Kluyveromyces lactis*. The response surface methodology (RSM) was also applied to optimize the medium components for the production of XynB secreted by the recombinant *K. lactis*. The secretion level (102 mg/l) and enzyme activity (49 U/ml) of XynB in the optimized medium (yeast extract, lactose, and urea; YLU) were much higher than those (56 mg/l, 16 U/ml) in the original medium (yeast extract, lactose, and peptone; YLP). The secretory efficiency of mature XynB was also improved when using the YLU medium. When the mRNA levels of 13 characterized secretion-related genes in the *K. lactis* cultured in YLP and YLU were detected using a semiquantitative RT–PCR method, the unfolded protein response (UPR)-related genes, including *ero1*, *hac1*, and *kar2*, were found to be up-regulated in the *K. lactis* cultured in YLU. Therefore, the nutrient ingredients, especially the nitrogen source, were shown to have a significant influence on the XynB secretory efficiency of the host *K. lactis*.

Keywords: Thermostable xylanase B, *Kluyveromyces lactis*, overexpression, response surface methodology, nitrogen source, secretion-related genes

Over the past decade, more than 40 heterologous proteins with different origins have been efficiently produced by *Kluyveromyces lactis* [18]. As a yeast host, *K. lactis* has several advantages: enzymes from *K. lactis* have a GRAS (generally regarded as safe) status that permits their use in food and feed applications, *K. lactis* can readily grow to a high density on inexpensive lactose-based media, and *K. lactis* does not require the explosion-proof fermentation equipment needed for the large-scale growth of methylotrophic yeasts. In addition, the easy genetic manipulation of *K. lactis* and the availability of its fully sequenced genome [5] have stimulated its use in research on secretion and glycosylation pathways [18]; the heterologous proteins expressed by *K. lactis* are often targeted by its secretory pathways for secretion into the culture media, thereby simplifying the downstream purification process; and *K. lactis* exhibits a relatively higher secretion efficiency than the conventional yeast *Saccharomyces cerevisiae* [18]. Consequently, many efforts have been made to elevate the protein production and secretion level of *K. lactis* [14] at various major points, including protein synthesis, folding, modification, intracellular trafficking, and exocytosis. Although most of these approaches have been successful in producing higher levels of particular proteins, the results nonetheless tend to be protein-specific [7]. Thus, identifying the key factors affecting the secretory capacity of *K. lactis* could provide additional targets for secretion pathway optimization.

β-1-4-Endoxylanases hydrolyze the β-1-4-glycosidic linkages of the xylan backbone to produce short-chain xylooligosaccharides of varying lengths, making them the key enzymes for the complete degradation of xylan [8]. Thus, the application of xylanases in biotechnological processes has received increased attention over the last few decades in the food and feed industries, and particularly the pulp and paper industry. As the pretreatment of pulp with xylanase optimally requires a high temperature and alkaline pH, this requires xylanases that are active and stable under these conditions [2]. A highly thermostable endoxylanase, XynB, which remains stable between pH 5.0 and 11.4, and at temperatures up to 100°C within a pH range of 7.0 to 8.5, has already been identified from the hyperthermophilic bacterium *Thermotoga maritima* MSB8 [23]. However, although the *xynB* gene has been

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cloned and expressed in *Escherichia coli* [26] and *Pichia pastoris* [24], the secretion target yield was very limited. In other studies, a family11 *xynA* structural gene from the extremely thermophilic anaerobe *Dictyoglomus thermophilum* Rt46B.1 [20], a *Thermotoga maritima* FjSS3b.1 *xynA* gene [21], and a putative *xyn11A* gene from *Bacillus halodurans* [22] have also been successfully expressed and secreted in *K. lactis*. However, the family10 *xynB* gene has not yet been expressed in *K. lactis*. Furthermore, XynB is thermostable and can be successfully expressed and secreted in various eukaryotic hosts, such as the yeasts *Pichia pastoris* [24] and *Hansenula polymorpha* (unpublished work), and the filamentous fungus *Aspergillus niger* [25]. Therefore, its characteristics make *Thermotoga maritima* XynB a good model system for exploring the secretory mechanism of eukaryotic heterologous protein expression platforms.

Accordingly, this study examined the heterologous expression and secretion of recombinant XynB of *T. maritima* in *K. lactis*. The medium components of the *K. lactis* system were also optimized for high-level laboratory-scale xylanase expression using the response surface methodology (RSM), an efficient statistical method. The secretory efficiencies of XynB by *K. lactis* cultivated in the initial medium and optimized medium were then compared, which also revealed the influence of the nutrient ingredients on the transcription levels of certain secretion-related genes in *K. lactis*.

Materials and Methods

Strains and Media

Escherichia coli (*E. coli*) DH5α was used for the storage and propagation of the plasmids. *K. lactis* strain GG799, included in the *K. lactis* Protein Expression Kit (New England Biolabs, USA), was used as the host for the expression of the *Thermotoga maritima* xylanase B (m$xynB_{(64)}$) gene (AY340789). An LB medium was used for the storage and culture of *E. coli*, whereas YPD (1% yeast extract, 2% peptone, 2% glucose), YPL (1% yeast extract, 2% peptone, 2% lactose; as recommended by the Instruction Manual of the *K. lactis* Protein Expression Kit), YLP (1% yeast extract, 2% lactose, 1.5% peptone; optimized medium based on orthogonal test), and YLU (1.2% yeast extract, 2.6% lactose, 0.5% urea; optimized medium based on RSM) were used to culture the yeast *K. lactis.* A YCB medium (New England Biolabs) with 5 mM acetamide was used when selecting the recombinant *K. lactis*.

Construction of *xynB* Expression Vector and Recombinant Yeast

The m$xynB_{(64)}$ gene was cloned previously in our laboratory [24]. It was cloned into the pKLAC1 vector (New England Biolabs) according to the manufacturer's instructions. The expression vector pKLAC1-$mxynB_{(64)}$ was linearized by *Sac*II digestion before transformation, and the integrative transformation of *K. lactis* GG799 was performed by electroporation, as described by W'esolowski-Louvel *et al.* [19]. The positive transformant colonies were then selected based on growth on YCB agar plates with 5 mM acetamide for 5 days at 30°C, and confirmed by a whole-cell PCR, as directed by the instruction manual.

Expression of Recombinant XynB and Determination of Xylanase Activity

The integrative transformants of *xynB* were isolated and precultured in a YPD medium, while shaken (200 rpm) at 30°C, until reaching an OD_{600} of 6~8. Thereafter, 2.5 ml of this seed culture was inoculated into a flask containing 50 ml of YPL, YLP, or YLU and shaken (200 rpm) for 4 days at 30°C. The biomass was determined by measuring the optical density (OD) with a spectrophotometer (Lambda Bio35, Perkin Elmer) using an absorbance of 600 nm. One OD unit corresponded to a dry weight (DW) of 0.385 mg/ml. A crude extract of the secreted enzyme was obtained by centrifugation of the fermentation broth at 16,000 ×*g* for 1 min. The xylanase activity was then assayed quantitatively using 1.15% RBB-xylan (Remazol Brilliant Blue-xylan; Sigma) as the substrate [24]. The standard assay mixtures (100 µl) consisted of 50 µl of 1.15% RBB-xylan, 25 µl of a 200 mM MES buffer, pH 6.5, and 25 µl of the enzyme solution. After incubation for 10 min at 70°C, the reaction was stopped by the addition of 200 µl of 100% ethanol, which precipitated the residual substrate, and the mixture was kept at room temperature for at least 15 min. The mixture was then centrifuged at 16,000 ×*g* for 5 min and the absorbance of the supernatant measured at 595 nm against the respective substrate blank. One unit of xylanase activity was defined as the amount of enzyme that liberated the Remazol Brilliant Blue dye from the substrate, causing a 1 OD increase in the reaction mixture per minute under the assay conditions described above [25, 26].

SDS–PAGE and Zymogram Analysis

The SDS–PAGE was performed according to the method of Laemmli [9] using 12% gels. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250, and the zymogram analysis was performed as described by Nakamura *et al.* [12], with a slight modification. The samples were electrophoresed on a 12% SDS polyacrylamide gel containing 0.1% birchwood xylan (Sigma). The gel was then washed four times for 15 min at 4°C in a 50 mM MES buffer (pH 6.5) [the first two washes contained 25% (v/v) isopropanol] to remove the SDS and renature proteins in the gel, and further incubated in the buffer for 10 min at 70°C. Thereafter, the gel was soaked in a 0.1% Congo red solution for 15 min at room temperature and washed with 1 M NaCl until the excess dye was removed from the active band. The gel was then incubated in 0.5% acetic acid, which turned the background dark blue and revealed clear zones in areas exposed to xylanase activity.

Optimization of Culture Medium Through Response Surface Methodology

The RSM was applied to optimize the medium components for XynB secretion. A central composite design (CCD) was processed to obtain a quadratic model, with xylanase activity as the response. The natural values and coded levels for the variables investigated are shown in Table 1. The variables were coded according to the following equation:

$$X_i=(x_i-x_{i0})/\delta_i \quad (1)$$

Table 1. Factors and coded values of central composite design.

Factors	Levels of factors				
	−1.682	−1	0	1	1.682
Yeast extract (x_1, g/l)	5.272	8	12	16	18.728
Lactose (x_2, g/l)	13.18	20	30	40	46.82
Urea (x_3, g/l)	0.636	2	4	6	7.364

where X_i is the coded value, x_i is the corresponding natural value, x_{i0} is the natural value in the center of the domain, and δ_i is the increment of x_i corresponding to 1 unit of X_i.

The response variable was fitted using a second-order model in order to correlate the response variable to the independent variables. The general form of the second-degree polynomial equation was as follows:

$$Y=\beta_0+\Sigma\beta_i X_i+\Sigma\beta_{ii}X_i^2+\Sigma\beta_{ij}X_iX_j \quad (2)$$

where Y is the measured response, β_0 is the intercept term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interactive coefficient. The experimental design is shown in Table 2. Statistical significance was determined by the F-value and P-value. An analysis of variance (ANOVA) was performed using SPSS (Statistical Program for Social Sciences), and the polynomial regression of the experimental data was performed using MATLAB7.1 (Mathworks Inc., MA, USA).

Table 2. Experimental design and results of central composite design.

Run	X_1	X_2	X_3	Y (xylanase activity, U/ml)	Y predicted (xylanase activity, U/ml)
1	−1	−1	−1	28.86	28.36
2	1	−1	−1	24.72	22.60
3	−1	1	−1	2.22	−2.52
4	1	1	−1	21.02	24.67
5	−1	−1	1	40.38	34.32
6	1	−1	1	25.16	27.49
7	−1	1	1	2.23	1.93
8	1	1	1	29.97	28.06
9	−1.682	0	0	1.73	7.46
10	1.682	0	0	26.91	24.59
11	0	−1.682	0	25.93	28.54
12	0	1.682	0	2.25	3.05
13	0	0	−1.682	28.33	29.38
14	0	0	1.682	34.89	37.25
15	0	0	0	41.52	39.93
16	0	0	0	39.30	39.93
17	0	0	0	42.66	39.93
18	0	0	0	42.66	39.93
19	0	0	0	38.49	39.93
20	0	0	0	35.55	39.93

Preparation of Secreted and Intracellular Proteins of Recombinant *K. lactis*

For the cell fractionation, 1 ml of the *K. lactis* culture was harvested by centrifugation (16,000 ×*g*, 5 min) at 4°C, and the supernatant and cells were processed separately. The supernatants were stored at −20°C and melted on ice before analysis. Meanwhile, the cell pellets were washed once with PBS (0.1 M phosphate buffer solution, pH 7.4) and resuspended in a mixture of 800 µl of PBS and 200 µl of zymolyase (Kirin, Japan). After a 2 h incubation at 35°C, the cells were lysed based on 10 min of vigorous vortexing with an equal volume of acid-washed glass beads (Sigma). The mixture was then incubated for 30 min at 80°C to remove any non-thermostable proteins. Thereafter, the precipitated protein was isolated by centrifugation (5 min at 16,000 ×*g*) in a microcentrifuge, and the supernatant was used to analyze the intracellular mature XynB.

Western Blotting and Protein Quantification

The quantification of the secreted and intracellular XynB levels of the recombinant *K. lactis* was performed as described previously [7], with a slight modification. The cell-free supernatant or cell lysate was electrophoresed by 12% SDS–PAGE, and the proteins were then transferred onto a polyvinylidene difluoride membrane (Millipore) and probed with anti-XynB antibodies raised in rabbits (Beijing Protein Institute, China), followed by a horseradish peroxidase-conjugated anti-rabbit antibody (Sigma). The immunodetection was performed with an enhanced chemiluminescence kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. For a quantitative comparison of the relative XynB concentrations, the average band intensities on developed film were quantified using the Bio-Rad Quantity One program. The slopes of the plotted lines for the intensity versus the exposure time were then used to compare the relative XynB levels. A known concentration of purified XynB was used to obtain the absolute XynB level for each sample. The experiments were all performed in triplicate.

Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT–PCR)

A semiquantitative RT–PCR was performed to evaluate the differences in the mRNA levels of the genes included in the protein secretion process of *K. lactis* cultured in YLP versus YLU media. The total RNA extraction was performed using a TRIzol Reagent (Invitrogen), as described previously [3], and quantified by spectrophotometry at 260 nm. Any contaminating genomic DNA was removed using RNase-free DNase I (TaKaRa). The first-strand cDNA was then synthesized by reverse transcription (RT reaction) in a final volume of 25 µl with 2 µg of total RNA, 1 µl of oligodT primer, 0.5 mM of dNTPs, and 200 U of M-MLV reverse transcriptase H minus (Promega). The reaction mixture was incubated for 60 min at 42°C and then used directly as the template. The PCR amplification was carried out for 5 min at 95°C, followed by 28 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The sequences for the secretion-related genes of *K. lactis* were obtained from the NCBI (http://www.ncbi.nlm.nih.gov). The primers are listed in Table 3. Equal volumes of the PCR amplification products were loaded on a 1% agarose gel. The gels were then stained with ethidium bromide (10 µg/ml) and photographed on top of a 280 nm UV light box. The bands that did not have an oversaturated intensity were then analyzed using the Bio-Rad Quantity One program. To normalize the sample-to-sample variation in the RT and PCR efficiency, relative values were obtained by comparing the intensities of the detected gene bands versus the

Table 3. Genes and primers used in semiquantitative RT–PCR.

Gene	Accession No.	Primer
act1	XM_453299	F: 5'-TTGGACTTCGAACAAGAAATGCAAA-3' R: 5'-GCGGTAATTTCCTTTTGCATTCTTT-3'
bmh1	XM_455629	F: 5'-TGTTGCTTACAAGAACGTTATTGGT-3' R: 5'-AAGGTCAAGTTATCTCTCAACAATT-3'
cog6	XM_455261	F: 5'-GGTTTGTAGATTATTTGTCCAAACT-3' R: 5'-AAGATACATGTCGTAATCAAGCTCA-3'
cup5	XM_454966	F: 5'-ATGAGTGATTTGTGTCCAGTGTAT-3' R: 5'-GTTCAACAATAGGGCAACAATCAAA-3'
dal5	XM_453200	F: 5'-TCAAGGATGTTAGAACTTGGTTGTA-3' R: 5'-CGATGTAAATAAGAACGATAATGAC-3'
ero1	CR_382124	F: 5'-CTGGATATGCTACTAGTTTGAAGAT-3' R: 5'-AGTTTATTGAGCTTTAGTACAGTGT-3'
hac1	XM_455488	F: 5'-AGGACCATTATTCTCATTCAAGTCA-3' R: 5'-GATTGGACTTATTATATCTGATGGT-3'
imh1	XM_452969	F: 5'-TAGCTCCAAAGAATCTGAATATAAG-3' R: 5'-CTAAGTCCTTAGTTTTACTGATTTG-3'
kar2	XM_453488	F: 5'-ATTATTGTCTATGATTTGGGTGGTG-3' R: 5'-ATTTTTTGGTTGGAATTGCAGTGTT-3'
kin2	XM_455957	F: 5'-TGTATTAACAACTTCTGCTGCCAAT-3' R: 5'-TCATTAATAACAATAGAAGGATGCC-3'
pdi1	AJ243958	F: 5'-AATACGAATCTCACTTTGTTGACTT-3' R: 5'-TGGCAATCAAGACCTTATCTTTCAA-3'
ssa3	XM_454878	F: 5'-GACAAAGGATAACAACTTATTGGGT-3' R: 5'-TTTTGTCTTTCCTTGTATTCATCTG-3'
sse1	XM_455059	F: 5'-TAAGTGGGAAGTTAATGGTGTTCAA-3' R: 5'-AAGCTTGCTTCTTTTCTTCTTCCTT-3'
sso2	CR382123	F: 5'-AAGGTTTTTGAAGGCCATTCAAGAT-3' R: 5'-GACTTCACAGCTTTATTTGTATGAC-3'

control *actin* product bands. The PCR amplifications were performed in triplicate, and the average values compared by a Student *t*-test using the SPSS (Statistical Program for Social Sciences).

RESULTS

Construction of Recombinant *K. lactis* pKLAC1-*mxynB*$_{(64)}$

K. lactis GG799 was transformed with pKLAC1-*mxynB*$_{(64)}$. After 5 days of growth on the selection medium, four individual transformant clones were confirmed by a PCR and then cultured in a liquid YPL medium. When using RBB-xylan as the substrate, xylanase activity was detected in the supernatant of all the confirmed transformants, but not in the GG799 control. The transformant with the highest activity, named GKX31, was then selected for the subsequent experiments.

Expression and Secretion of Xylanase from Recombinant *K. lactis*

The *xynB* gene is controlled by the *LAC4* promoter, which is inducible by lactose. The xylanase activity of the culture supernatant of recombinant *K. lactis* GKX31 reached 16.3 U/ml after 96 h of culture in a shaking flask when using YPL as the fermentation medium. The supernatant of the fermentation broth after 96 h was also treated at 80°C for 30 min. The supernatants of both the fermentation broth and the heat-treated broth were then loaded onto an SDS–PAGE gel, and after electrophoresis, a band of ~40 kDa was observed for the GKX31 transformant and its heat-treated broth, but not for GG799 (Fig. 1A, arrow). To confirm the xylanase activity of the 40 kDa protein, a zymogram analysis was performed, where clear zones in the dark background indicated exposure to xylanase activity (Fig. 1B). Xylanase activity was retained even when the fermentation broth was heated at 80°C.

Optimization of Fermentation Medium Using Response Surface Methodology

In preliminary experiments, an orthogonal experiment was employed to optimize the YPL medium, as recommended by the kit instruction manual. However, the optimized YLP medium only had a minimal effect on increasing the XynB production by *K. lactis* GKX31 (data not shown). Therefore, the effects of different nitrogen sources on the XynB

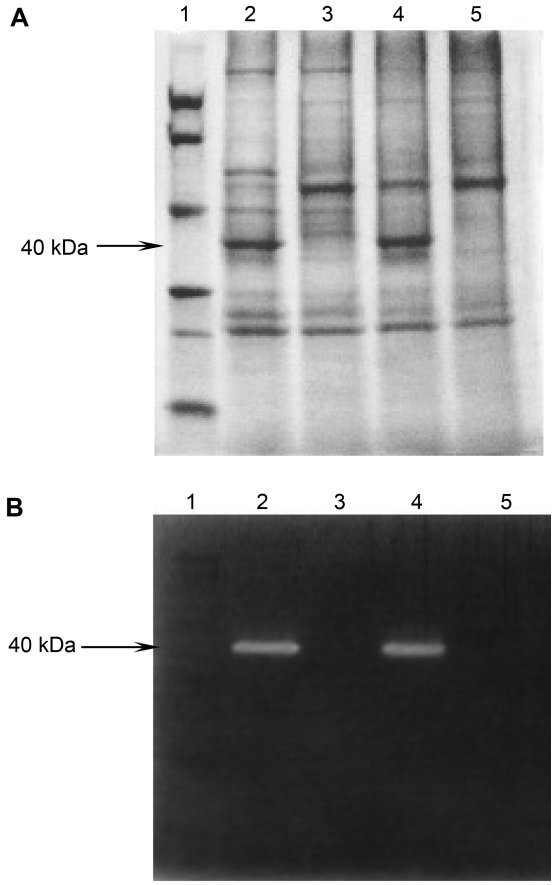

Fig. 1. SDS–PAGE **(A)** and zymogram **(B)** analysis of XynB secreted by recombinant *K. lactis* GKX31.
Lane 1: Molecular mass markers (97, 66, 43, 31, 20 KDa); Lane 2: Secreted proteins of *K. lactis* GKX31; Lane 3: Proteins secreted by *K. lactis* GG799 (negative control). Lane 4: Proteins in lane 2 after heating to eliminate non-thermostable proteins. Lane 5: Proteins in lane 3 after heating to eliminate non-thermostable proteins.

production by *K. lactis* GKX31 were investigated in media with 2% lactose and 1% yeast extract. Based on the results, as presented in Fig. 2, urea was selected instead of the original peptone as the nitrogen source in YLP. The concentrations of the yeast extract (x_1=12 g/l), lactose (x_2= 30 g/l), and urea (x_3=4 g/l) were then chosen as the central point of the CCD. Twenty sets of tests were performed: 2^3 full factorial, six star points, and six central points; the results are shown in Table 2. A full quadratic model was obtained from a regression analysis of the data from the CCD experiment:

$$Y=39.9334+5.0914X_1-7.5786X_2+2.3383X_3-8.4499X_1^2-8.5312X_2^2-2.3392X_3^2+8.2392X_1X_2-0.2673X_1X_3-0.3773X_2X_3 \quad (3)$$

where Y is the predicted response, the XynB activity (U/ml) in the supernatant of the fermentation broth, and X_1, X_2, and X_3 are the coded values for the yeast extract, lactose, and urea concentrations, respectively.

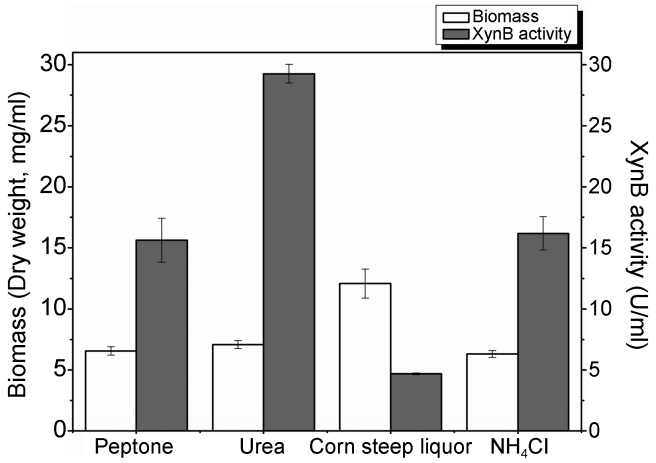

Fig. 2. Effects of different nitrogen sources on cell growth (dry weight, mg/ml) and XynB activity (U/ml) of recombinant *K. lactis*.

The results of the analysis of variance (ANOVA) for the quadratic regression model are shown in Table 4. The regression model was highly significant, as evident from the calculated F-value (22.82) and very low probability value (P<0.0001). In addition, the value of the multiple correlation coefficient R (0.9765) was close to 1, indicating a high degree of correlation between the observed and predicted values, whereas the value of the determination coefficient R^2 (0.9532) indicated that 95.32% of the variability in the response could be explained by the model. Furthermore, the model showed a statistically insignificant lack of fit, as evident from the lower calculated F-value (3.56) than the tabulated F-value ($F_{0.05(5,5)}$=5.05), even at a 0.05 level. Thus, the model was found to be adequate for prediction within the range of the variables employed.

The significance of the regression coefficients was evaluated by a t-test (Table 5), and the results showed that all the quadratic terms, linear coefficients of the three factors, and interaction between X_1 (yeast extract) and X_2 (lactose) had a significant effect on the xylanase activity. In addition, a 2D contour plot (Fig. 3) provided a visual

Table 4. ANOVA of regression model.

Source	SS	DF	MS	F-value	$P>F$
Model	3,657.8401	9	406.4266	22.8237	<0.0001
Residual	178.0717	10	17.8072		
Lack of fit	139.0429	5	27.8085	3.5625	0.0947
Pure error	39.0288	5	7.8057		

R=0.9765; R^2=0.9536; SS, sum of squares; DF, degrees of freedom; MS, mean square.

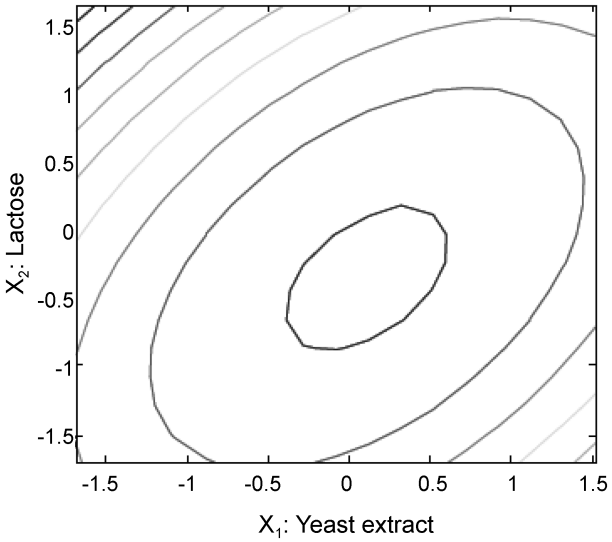

Fig. 3. Contour plot of combined effects of yeast extract and lactose on enzyme activity of recombinant XynB.
Concentrations on the x axis are given in coded forms as listed in Table 1.

interpretation of the interaction between the yeast extract and lactose concentrations, where the shape of the contour plot indicated a positive interaction between these variables: the xylanase activity increased with a simultaneous increase of both factors.

The optimal values for each independent variable in the coded form were X_1=0.1, X_2=−0.41, and X_3=0.55, whereas the actual values were 12.4 g/l yeast extract, 25.9 g/l lactose, and 5.1 g/l urea. The model predicted that the recombinant xylanase activity would reach 42.3 U/ml when using YLU, the optimized medium, and this optimized value was validated in triplicate shaking-flask experiments, where the mean value of xylanase activity was 49 U/ml, which was reasonably close to the predicted value. Thus, the model was shown to be adequate.

Optimized Medium Increased XynB Secretion Efficiency of *K. lactis*

K. lactis GKX31 from the same seed culture was inoculated into YLU and YLP media. XynB expression was then induced using both media for 96 h, until the extracellular enzyme activity stopped increasing. The biomass, extracellular and intracellular xylanase activities, secretion, and intracellular yields of mature XynB were all recorded during the 96 h. As shown in Fig. 4A, the cell growth profiles were similar for YLU and YLP. After 42 h, the biomass (expressed as dry weight, mg/ml) in YLU was a little higher than that in YLP. To compare the XynB secretion efficiency of the recombinant *K. lactis* cultivated in YLU and YLP, a time course analysis of the extracellular and intracellular xylanase activities, secretion, and intracellular levels of mature XynB was performed. Because of the biomass difference in YLU and YLP, the xylanase activities and XynB levels were expressed on a dry weight basis as U/mg DW and mg/g DW, respectively (Fig. 4B–4E). Whereas XynB was continuously secreted in both media, its extracellular activity and secretion level in YLU were much higher than those in YLP (Fig. 4B and 4C). In both media, the intracellular mature XynB reached a maximal yield during the initial stage of culture, and then decreased rapidly. After 30 h, the level of intracellular mature XynB decreased slowly in YLU, but increased slightly in YLP. At the end of the culture, the yield of intracellular mature XynB in YLP was slightly higher than that in YLU (Fig. 4D and 4E). When combining these factors, for much of the time-course, particularly after 30 h, the percentage of secreted XynB compared with the total mature XynB was higher in YLU (33–48%) than in YLP (18–35%), meaning that the secretion efficiency of XynB was improved by YLU. The highest production of XynB at 96 h, expressed as the volumetric yield, was 101.5 mg/l fermentation supernatant in YLU and 55.7 mg/l in YLP.

Table 5. Regression results of central composite design.

Factor	coefficient	P-value
Intercept	33.9334	<0.0001*
X_1	5.0914	0.0004*
X_2	−7.5786	<0.0001*
X_3	2.3383	0.0453*
X_1^2	−8.4499	<0.0001*
X_2^2	−8.5312	<0.0001*
X_3^2	−2.3392	0.0405*
X_1X_2	8.2392	0.0001*
X_1X_3	−0.2673	0.8548
X_2X_3	−0.3773	0.7960

*Statistically significant at 95% confidence level.

Differences in mRNA Levels of *K. lactis* Secretion- and Stress-Related Genes Expressed in YLU and YLP Cultures

In *P. pastoris*, the expression levels of 13 secretion- and stress-related genes have been found to change according to the heterologous protein secretion [6]. Thus, a semiquantitative RT–PCR was performed to compare the mRNA levels of these genes in *K. lactis* when using YLU and YLP as the culture media. As seen from Table 6, the mRNA levels of the unfolded protein response related genes *hac1*, *ero1*, and *kar2* were 2.4-, 1.7-, and 1.5-fold higher, respectively, in the YLU culture than in the YLP culture. The mRNA levels of *kin2*, *sso2*, *pdi1*, and *sse1* were also 1.4- to 1.2-fold higher in the YLU culture than in the YLP culture. The mRNA levels of the other detected genes showed no significant up-regulation in the YLU culture. The three genes above the threshold (±1.5-fold regulation) are shown in Fig. 5.

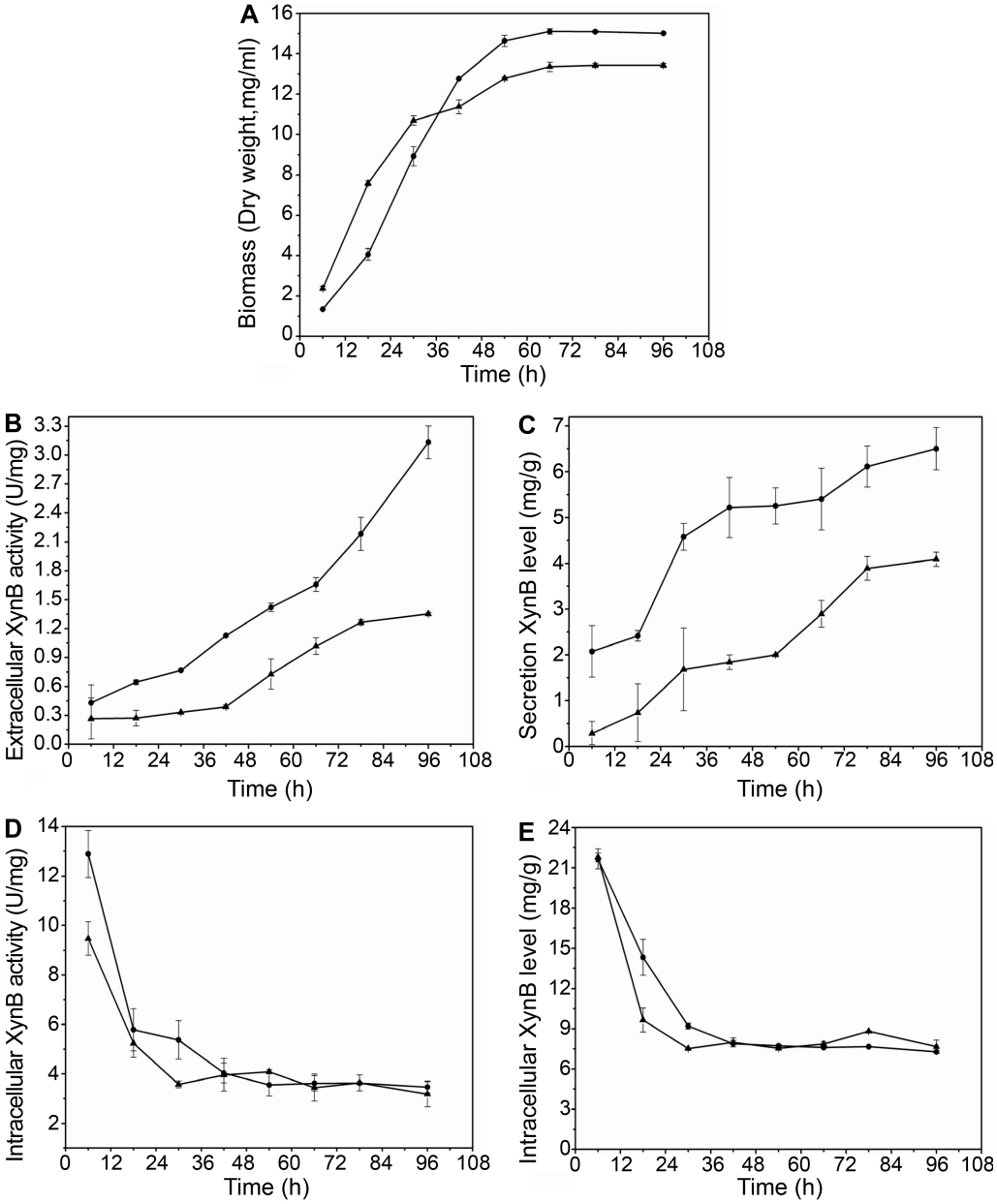

Fig. 4. Dynamics of biomass, extracellular xylanase activity, XynB secretion level, intracellular xylanase activity of mature protein, and intracellular mature XynB level of *K. lactis* GKX31 cultured in YLP (▲) and YLU (●).

A. Growth curves of recombinant *K. lactis* cultured in YLP and YLU. Biomass is expressed as dry weight (mg/ml). **B**. Dynamics of extracellular xylanase activities (dry weight basis) in YLP and YLU. Unit of xylanase activity is expressed as per dry weight activity (U/mg). **C**. Dynamics of XynB secretion levels (dry weight basis) in YLP and YLU. The XynB level was quantified by comparing the band intensity of the protein of interest on the developed PVDF film with that of a known concentration of purified XynB on the same film using the Quantity One program. Unit of XynB level is expressed as per dry weight yield (mg/g). **D**. Dynamics of intracellular xylanase activities (dry weight basis) in YLP and YLU. Unit of xylanase activity is expressed as per dry weight activity (U/mg). **E**. Dynamics of intracellular mature XynB levels (dry weight basis) in YLP and YLU. The cell lysate was incubated at 80°C for 30 min, centrifuged, and the supernatant containing the mature XynB used to quantify the intracellular mature XynB levels. Unit of XynB level is expressed as per dry weight yield (mg/g). For all panels, triplicate independent samples were used for each data point and associated error bar.

DISCUSSION

The endoxylanase XynB from *T. maritima* MSB8 displays a high thermal and pH stability, giving it great potential for industrial application, particularly in the pulp and bleach industries [23]. Nonetheless, there have been no reports of its industrial-scale production, perhaps due to the low yield of this enzyme. Based on secretory expression, the current authors previously expressed this XynB in *P. pastoris*, but the potential for industrial-scale manufacture was restricted

Table 6. Mean improvement of mRNA levels of secretion-related genes in YLU culture relative to that in YLP culture.

Gene	Protein localization	Mean improvement	*P*-value[a]	Statistical significance[b]
hac1	ER	2.4	0.025074	*
ero1	ER	1.7	0.023341	*
kar2	ER	1.5	0.002295	**
kin2	Cytoplasma face	1.4	0.11922	
sso2	Cytoplasma	1.4	0.21952	
pdi1	ER	1.3	0.040096	*
sse1	ER	1.2	0.017223	*
ssa4	Cytoplasma	1.1	0.062143	
cog6	Golgi apparatus	1.0	0.030982	*
imh1	Cytoplasma	1.0	0.192635	
bmh2	Cytoplasma	1.0	0.21349	
cup5	Cytoplasma	0.9	0.254825	
sec31	Vesicle	0.8	0.028547	*

[a]Statistical significance of the mean improvement of the mRNA level of each detected gene determined by the Student *t*-test.
[b]*, $P<0.05$; **, $P<0.01$.

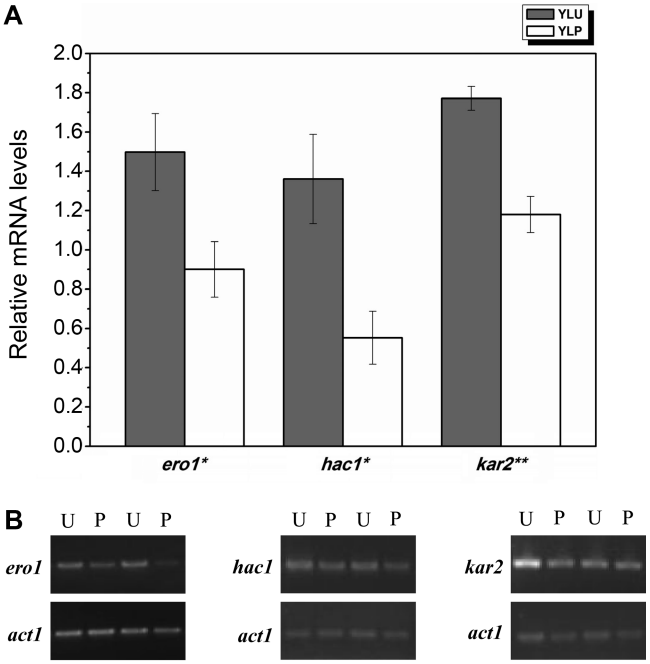

Fig. 5. Differences in the relative transcription and mRNA level of *ero1*, *hac1*, and *kar2* genes.
A. Schematic representation of differences in the relative transcription levels of *ero1*, *hac1*, and *kar2* of *K. lactis* cultured in YLU and YLP. *, Statistically significant at the 95% confidence level; **, Statistically significant at the 99% confidence level. **B**. Results of semiquantitive RT–PCR, showing differences in the mRNA levels of *ero1*, *hac1*, and *kar2* of *K. lactis* cultured in YLU (U) and YLP (P).

by a low secretory xylanase activity (5.6 U/ml [24]). *H. polymorpha*, another well-established yeast expression system, was also employed to express this XynB, but the secreted protein showed a lower xylanase activity than that expressed in *P. pastoris* (unpublished data). In contrast, in this study, the XynB protein expressed in *K. lactis*, a yeast host with a strong record in protein secretion, showed a high secretion yield and enzyme activity.

For the overproduction of an enzyme, medium optimization of the host strain is the primary task. In the present study, RSM, an efficient and widely used statistical method, was applied to optimize the XynB production by the recombinant *K. lactis*. As a result, the extracellular XynB activity increased approximately 3-fold from the initial shake-flask fermentation of the recombinant *K. lactis* using YLP to the experiment using YLU. In addition, the RSM results revealed that the yeast extract, urea, and nitrogen composition of YLU were the main factors with a positive effect on the XynB secretion.

The time-course analysis of the XynB level on a dry weight basis revealed that the optimized medium improved the secretory efficiency of XynB in *K. lactis* (Fig. 4). Notwithstanding, over 50% of the mature XynB remained in the cells with YLU cultivation, indicating that a further yield of at least 100 mg/l of mature XynB can reasonably be expected through secretory pathway optimization of this yeast host. Successful high-level secretion of recombinant proteins can be limited at several distinct steps, including folding, disulfide bridge formation, glycosylation, transport within the cell, and release from the cell. For the thermostable XynB in this study, the mature form of the protein means that it has been properly folded to its compact conformation and its prepro leader sequence has been completely processed by Kex2 protease in *tran*s Golgi. The fact that a significant amount of mature XynB remained in the cells implies that successful secretion of the mature protein was blocked in the later stage of the secretion pathway, such as vacuolar transportation, the secretory vesicle fusion at the plasma membrane or space between the plasma membrane and the cell wall.

Recent studies have indicated that many protein products exert severe stress on the host cell when overexpressed, thereby limiting the potential yield. Thus, a detailed understanding of the physiological responses to such stress could open the gate to engineering host cells that can better cope with stress factors [11]. In the previous work, overexpression of the secretion-related genes in *K. lactis* was found to enhance the release of heterologous proteins outside the cell [1, 10, 16, 17]. Furthermore, recent transcriptomic [6] and proteomic studies [18] have provided insights on the cellular response of yeast to the overexpression of heterologous proteins. Gasser *et al*. [6] identified 13 genes, involved in different stages of the secretory pathway, that were up-regulated in a heterologous protein expression strain versus a non-expression strain of *P. pastoris*. However, information on the cellular response of yeast expressing heterologous proteins to the nutritional conditions remains limited. In the present study,

the conspicuous difference between YLP and YLU was the nitrogen composition, which was proved by RSM as the main factor affecting XynB production. When comparing the mRNA levels of *K. lactis* homologs of the above-mentioned genes in YLP and YLU cultures of the *K. lactis* strain expressing heterologous XynB, three previously characterized UPR-related genes, *hac1*, *ero1*, and *kar2*, showed higher transcriptional levels in the YLU culture than in the YLP culture. The *hac1* gene encodes transcription factor Hac1, which induces the genes of the chaperones and enzymes involved in the secretion process, whereas *ero1* and *kar2* encode the chaperones that mediate the protein folding in the endoplasmic reticulum (ER) [4, 13]. Although the secretory efficiency of mature XynB in the YLU culture was higher than that in the YLP culture, the mRNA levels of the detected genes involved in the later stage of the secretory pathway were hardly different in the *K. lactis* cultivated in the two media. As the complex mechanism of heterologous protein secretion by yeast remains unclear, other helper genes may also be involved in the later stage of the secretory pathway. Nonetheless, the present results demonstrated that the optimized medium still influenced the expression of the heterologous protein and up-regulated the genes involved in UPR, which affect almost every stage of the secretory pathway [15]. Thus, studies utilizing optimized media will advance the current understanding of the mechanism of heterologous protein secretion by *K. lactis* and facilitate new strategies to enhance the production capabilities of these cells.

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REFERENCES

1. Bao, W. G. and H. Fukuhara. 2001. Secretion of human proteins from yeast: Stimulation by duplication of polyubiquitin and protein disulfide isomerase genes in *Kluyveromyces lactis*. *Gene* **272:** 103–110.
2. Beg, Q. K., M. Kapoor, L. Mahajan, and G. S. Hoondal. 2001. Microbial xylanases and their industrial applications: A review. *Appl. Microbiol. Biotechnol.* **56:** 326–338.
3. Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
4. Dorner, A. J. and R. J. Kaufman. 1994. The levels of endoplasmic reticulum proteins and ATP affect folding and secretion of selective proteins. *Biologicals* **22:** 103–112.
5. Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, *et al.* 2004. Genome evolution in yeasts. *Nature* **430:** 35–44.
6. Gasser, B., M. Sauer, M. Maurer, G. Stadlmayr, and D. Mattanovich. 2007. Transcriptomics-based identification of novel factors enhancing heterologous protein secretion in yeasts. *Appl. Environ. Microbiol*. **73:** 6499–6507.
7. Huang, D., P. R. Gore, and E. V. Shusta. 2008. Increasing yeast secretion of heterologous proteins by regulating expression rates and post-secretory loss. *Biotechnol. Bioeng.* **101:** 1264–1275.
8. Kulkarni, N., A. Shendye, and M. Rao. 1999. Molecular and biotechnological aspects of xylanases. *FEMS Microbiol. Rev.* **23:** 411–456.
9. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
10. Lodi, T., B. Neglia, and C. Donnini. 2005. Secretion of human serum albumin by *Kluyveromyces lactis* overexpressing KlPDI1 and KlERO1. *Appl. Environ. Microbiol.* **71:** 4359–4363.
11. Mattanovich, D., B. Gasser, H. Hohenblum, and M. Sauer. 2004. Stress in recombinant protein producing yeasts. *J. Biotechnol.* **113:** 121–135.
12. Nakamura, S., K. Wakabayashi, R. Nakai, R. Aono, and K. Horikoshi. 1993. Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M-1. *Appl. Environ. Microbiol.* **59:** 2311–2316.
13. Nishikawa, S. I., S. W. Fewell, Y. Kato, J. L. Brodsky, and T. Endo. 2001. Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. *J. Cell Biol.* **153:** 1061–1070.
14. Raimondi, S., E. Zanni, C. Talora, M. Rossi, C. Palleschi, and D. Uccelletti. 2008. SOD1, a new *Kluyveromyces lactis* helper gene for heterologous protein secretion. *Appl. Environ. Microbiol.* **74:** 7130–7137.
15. Travers, K. J., C. K. Patil, L. Wodicka, D. J. Lockhart, J. S. Weissman, and P. Walter. 2000. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101:** 249–258.
16. Uccelletti, D., S. Anticoli, and C. Palleschi. 2007. The apyrase KlYnd1p of *Kluyveromyces lactis* affects glycosylation, secretion, and cell wall properties. *FEMS Yeast Res.* **7:** 731–739.
17. Uccelletti, D., D. Staneva, S. Rufini, P. Venkov, and C. Palleschi. 2005. Enhanced secretion of heterologous proteins in *Kluyveromyces lactis* by overexpression of the GDP-mannose pyrophosphorylase, KlPsa1p. *FEMS Yeast Res.* **5:** 735–746.
18. van Ooyen, A. J., P. Dekker, M. Huang, M. M. Olsthoorn, D. I. Jacobs, P. A. Colussi, and C. H. Taron. 2006. Heterologous protein production in the yeast *Kluyveromyces lactis*. *FEMS Yeast Res.* **6:** 381–392.
19. W'esolowski-Louvel, M., K. D. Breunig, and H. Fukuhara. 1996. *In* K. Wolf (ed.). *Nonconventional Yeasts in Biotechnology: A Handbook*, pp. 139–202. Springer-Verlag, Berlin, New York.
20. Walsh, D. J. and P. L. Bergquist. 1997. Expression and secretion of a thermostable bacterial xylanase in *Kluyveromyces lactis*. *Appl. Environ. Microbiol.* **63:** 3297–3300.
21. Walsh, D. J., M. D. Gibbs, and P. L. Bergquist. 1998. Expression and secretion of a xylanase from the extreme thermophile, *Thermotoga* strain FjSS3B.1, in *Kluyveromyces lactis*. *Extremophiles* **2:** 9–14.

22. Wamalwa, B. M., G. Zhao, M. Sakka, P. M. Shiundu, T. Kimura, and K. Sakka. 2007. High-level heterologous expression of *Bacillus halodurans* putative xylanase Xyn11A (BH0899) in *Kluyveromyces lactis*. *Biosci. Biotechnol. Biochem.* **71:** 688–693.
23. Winterhalter, C. and W. Liebl. 1995. Two extremely thermostable xylanases of the hyperthermophilic bacterium *Thermotoga maritima* MSB8. *Appl. Environ. Microbiol.* **61:** 1810–1815.
24. Yang, M. H., Y. Li, G. H. Guan, and Z. Q. Jiang. 2005. High-level expression of an extreme-thermostable xylanase B from *Thermotoga maritima* MSB8 in *Escherichia coli* and *Pichia pastoris*. *Wei Sheng Wu Xue Bao* **45:** 236–240.
25. Zhang, J., J. Pan, G. Guan, Y. Li, W. Xue, G. Tang, A. Wang, and H. Wang. 2008. Expression and high-yield production of extremely thermostable bacterial xylanase B in *Aspergillus niger*. *Enzyme Microb. Technol.* **43:** 513–516.
26. Zhengqiang, J., A. Kobayashi, M. M. Ahsan, L. Lite, M. Kitaoka, and K. Hayashi. 2001. Characterization of a thermostable family 10 endo-xylanase (XynB) from *Thermotoga maritima* that cleaves *p*-nitrophenyl-beta-D-xyloside. *J. Biosci. Bioeng.* **92:** 423–428.