

## High-Level Heterologous Expression of *Bacillus halodurans* Putative Xylanase Xyn11A (BH0899) in *Kluyveromyces lactis*

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The putative *xyn11A* structural gene (BH0899) encoding a family-11 xylanase from alkaliphilic *Bacillus halodurans* strain C-125 was heterologously expressed in the yeast *Kluyveromyces lactis* CBS 1065 and secreted to a level of 156 µg/ml under selective culture conditions in shake flasks. The Xyn11A production level in shake flask cultures of *K. lactis* CBS 1065 was higher than that reported for other xylanase genes placed under the control of the regulated LAC4 promoter on a plasmid containing an entire sequence of pKD1 from *Kluyveromyces drosophilarius*. Recombinant Xyn11A was highly active over pH range from 3 to 10, with maximal activity around pH 7. The enzyme showed a specific activity of 628 U/mg-protein on birchwood xylan as substrate, but no cellulase or β-xylosidase activity.

**Key words:** *Bacillus halodurans*; *Kluyveromyces lactis*; xylanase; heterologous expression; secretion

Studies on alkaliphiles have led to the discovery of many enzymes of biotechnological interest. Several of these have been produced on an industrial scale.<sup>1)</sup> A variety of prokaryotic and eukaryotic organisms have been employed as heterologous expression hosts in attempts to improve the production of xylanases for biotechnological applications. Indeed, endoxylanases from several microorganisms have been expressed in yeast, mainly for biomass utilization, commercial prebleaching and bakery applications. Over 40 diverse heterologous proteins have, for instance, been efficiently produced with the yeast *Kluyveromyces lactis* as an expression host.<sup>2)</sup> Enzymes from *K. lactis* have GRAS (generally regarded as safe) status and may be used in feed and food applications.<sup>2)</sup> Emphasis is being laid on the development of suitable and/or improved xylanase-

producing recombinant yeast strains with a view to enhancing industrial enzyme production processes.<sup>3,4)</sup> In this study, a *Bacillus halodurans* C-125 genome sequence-derived open reading frame, BH0899, which encodes a putative endo-β-xylanase Xyn11A, was isolated and fused in frame with the *K. lactis* killer toxin secretion signal of a newly developed episomal pKD1-based vector for expression in *K. lactis*.<sup>5–7)</sup> Below we report high-level secretion of *B. halodurans* recombinant Xyn11A in *K. lactis* and its functional properties.

### Materials and Methods

*Strains, plasmids, media, and culture conditions.* *B. halodurans* C-125 was obtained from the Japan Collection of Microorganisms (JCM). Plasmids pET-28a (Novagen, Madison, WI) and pCWK:I (Pacific Gem Limited, Hamilton, New Zealand)<sup>5)</sup> were used as cloning and expression vectors respectively. *Escherichia coli* DH5α, used as a host for cloning, was transformed and cultured at 37 °C in Luria-Bertani medium supplemented with kanamycin (50 µg/ml) for plasmid selection.<sup>8)</sup> Wild-type *Kluyveromyces lactis* CBS 1065 (Centraalbureau voor Schimmelculturen, Utrecht, Netherlands) was used as the expression host. Strain CBS 1065 was electrotransformed and grown at 30 °C in YPD (1% yeast extract, 2% Bacto-peptone and 2% glucose), as reported by Sánchez *et al.*<sup>9)</sup> and Walsh *et al.*<sup>10)</sup> respectively. Yeast recombinants were selected for resistance to 200 µg/ml of G418 (Geneticin; Life Technologies, Gaithersburg, MD) as described elsewhere.<sup>10)</sup>

*Nucleic acid isolation, plasmid construction, and recombinant Xyn11A (rXyn11A) expression.* Genomic

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Abbreviations: BWX, birchwood xylan; CMC, carboxymethylcellulose; EKP, eucalyptus kraft pulp; OSX, oat-spelt xylan; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography

DNA was isolated from *B. halodurans*, as previously described.<sup>11)</sup> The cloning plasmid was constructed as follows: Oligonucleotide primers CxF 5'-AGTACCGGCCATGGGCAATACCT-3' and CxR 5'-TACGCTCGAGCCAAACCGTCACA-3' were designed on the basis of the coding sequences for the amino and carboxy termini of locus BH0899 to allow PCR amplification of a signal peptide-lacking derivative of the putative *xyn11A* gene from *B. halodurans* genomic DNA (DDBJ accession no. BA000004). *NcoI* (5' end) and *XhoI* (3' end) restriction endonuclease sites (underlined nucleotides in bold type) were incorporated in the primers to allow directional ligation of the PCR product into the same sites of pET-28a. PCR was performed using PCR master mix (Qiagen, Hilden, Germany) and a thermal cycler (BIO-RAD Laboratories, Hercules, CA) under the following conditions: one cycle of initial denaturation at 98 °C for 5 min; 26 cycles of denaturation, annealing, and extension at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min respectively; and one final extension cycle of 7 min at 72 °C. The fidelity of the new vector-insert cassette, named pET28a-*xyn11A*, was confirmed by bi-directional DNA sequencing. The yeast expression plasmid was constructed as follows: Primers CxM1F 5'-CAACGCGTAATACCTATTGGCAA-3', and Cel51R 5'-GTTACGCGTCGGATCTCAGT-3', were designed on the basis of the pET28a-*xyn11A* construct to allow PCR amplification of coding sequences for a C-terminal 6-His-tagged derivative of Xyn11A with *MluI* sites (underlined nucleotides in bold type) at both the 5' and the 3' end. PCR was performed under the conditions described above. The PCR product was digested with *MluI* and ligated into pCWK:I also digested with *MluI* to give an in-frame fusion with the killer toxin leader sequence of the vector and thus placed under the control of the LAC4 promoter and terminator sequences. The plasmid, named pCWK:I-*xyn11A6His*, provided a recombinant Xyn11A 6-His-tagged at the C-terminus (rXyn11A).

*K. lactis* CBS 1065 was transformed with the pCWK:I-*xyn11A6His* construct by electroporation (1,000 V, 25 µF, and 400 Ω settings on controller devices of a Bio-Rad GenePulser), and positive transformants were selected for polypeptide production in YPD medium. Four-d-old recombinant *K. lactis* CBS 1065 cell cultures were centrifuged (4,000 × g, 10 min) and the crude cell-free supernatant was divided into two portions. Portion 1 was extensively dialyzed against Britton-Robinson's universal buffer (50 mM phosphoric acid, 50 mM boric acid, and 50 mM acetic acid, pH 7.0) at 4 °C and used for extracellular xylanase activity assay against birchwood xylan (BWX; Sigma-Aldrich, St. Louis, MO). Portion 2 was used for the preparation of purified rXyn11A samples using a Ni-NTA resin column (Qiagen). The cell-free culture supernatant (1 liter) of *K. lactis* was dialyzed against sodium phosphate buffer (pH 7), then preconcentrated at 4 °C down to 25 ml using PEG20000. Aliquots of the concentrate containing the secreted

6His-tagged Xyn11A were then applied onto a Ni-NTA column, purified to apparent homogeneity and used in enzyme assays. Secretion of rXyn11A by *K. lactis* was verified on Azo-Xylan (Megazyme, Bray, IN) YPD agar plates. Culture supernatant and samples of purified rXyn11A were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 12.5% gels.<sup>12)</sup> The gels were stained with coomassie brilliant blue G250 (Bio-Rad, Hercules, CA) for protein visualization. The protein concentration was determined using Bradford reagent from Bio-Rad with BSA as the standard.

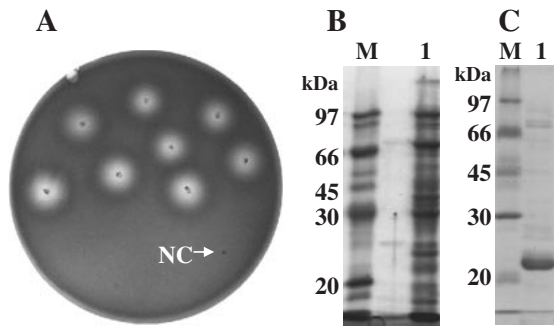
*Enzyme assays and analysis of reaction products by thin layer chromatography (TLC).* The substrate specificity of purified enzyme preparations was quantified by measuring released reducing sugar from BWX, carboxymethylcellulose (CMC, Sigma-Aldrich), or Avicel (Merck, Darmstadt, Germany) by the method of Somogyi-Nelson,<sup>13)</sup> with glucose or xylose as standard. Hydrolase activity on *p*-nitrophenyl β-D-xylopyranoside (PNPX, Sigma-Aldrich) was determined as described by Wood and Bhat.<sup>14)</sup> Kinetic parameters were determined using xylan from birchwood and oat spelts at pH and temperature conditions of maximal enzyme activity.

In the thin layer chromatography (TLC) assay, BWX, OSX, eucalyptus kraft pulp (EKP, the kind gift of T. Mitsunaga), or xylooligosaccharide (xylobiose[X2], xylotriase[X3], xyloetraose[X4], and xylopentaose[X5], 4 mg of each; Megazyme) was incubated with rXyn11A (30 µg) for 16 h in a 200 µl reaction mixture in Britton-Robinson's buffer (pH 7.1) at 40 °C. Degradation products were qualitatively determined on pre-coated sheets (Silica gel 60, Merck) with butanol-acetic acid-water (10:5:1, vol/vol/vol) and visualized as described elsewhere.<sup>15)</sup>

## Results

### *Construction of K. lactis recombinant and production of rXyn11A*

The *B. halodurans xyn11A* gene encodes 210 amino acid residues including a signal peptide, and Xyn11A is a single domain xylanase classified in family 11 of glycoside hydrolases ([on-line] the CAZy database, <http://afmb.cnrs-mrs.fr/CAZY/index.html>).<sup>16)</sup> The recombinant plasmid pCWK:I-*xyn11A6His* produces a chimeric polypeptide consisting of an N-terminal signal peptide of the *K. lactis* killer toxin, a family-11 catalytic domain of Xyn11A, and a 6-His tag. Electrotransformation of *K. lactis* CBS 1065 cells harvested in mid-exponential phase (absorbance at 600 nm = 1.4, 3 × 10<sup>7</sup> cells/ml of culture) was achieved with a 6.1 ms pulse time. Colonies of recombinant *K. lactis* strain CBS 1065 cells carrying the pCWK:I-*xyn11A6His* construct expressed and secreted xylanase activity in Azo-xylan YPD agar, in contrast to control *K. lactis* CBS 1065 cells transformed with the original pCWK:I plasmid (Fig. 1A and B). Strain CBS 1065 recombinants show-



**Fig. 1.** Production of rXyn11A by Recombinant *K. lactis* CBS 1065.

A, Azo-Xylan agar assay of the extracellular secretion of xylanase by *K. lactis* recombinants harboring pCWK:I-*xyn11A6His* and a negative control *K. lactis* transformant harboring pCWK:I (NC). B, SDS-PAGE assay of proteins expressed by a recombinant *K. lactis* CBS 1065 (pCWK:I-*xyn11A6His*) in YPD culture supernatant. Lanes: M, low molecular weight marker; 1, proteins in culture supernatant of the recombinant. C, SDS-PAGE of rXyn11A purified from culture supernatant of *K. lactis* CBS 1065 (pCWK:I-*xyn11A6His*). Lanes: M, low molecular weight marker; 1, recombinant Xyn11A. The sizes of molecular weight standards are indicated on the left.

ing large clear zones around colonies in Azo-xylan YPD agar were picked out and grown at 30 °C in shake flasks (120 rpm) for 4 d in YPD medium. The highest level of xylanase activity secreted into the culture medium was 98 U/ml (where U =  $\mu$ moles of xylose released from BWX per min under assay conditions).

#### Characterization of rXyn11A

Protein rXyn11A was verified to be present in these *K. lactis* culture supernatant samples after protein purification on Ni-NTA columns and subsequent fractionation by SDS-PAGE (Fig. 1C). A summary of the physicochemical constants, substrate specificities, and apparent  $K_m$  value (estimated from Lineweaver-Burk plots, data not shown) for rXyn11A on xylan from birchwood and oat speltis is presented in Table 1. The activity of the recombinant enzyme remained above

**Table 1.** Summary of Characterization of *B. halodurans* Recombinant Xyn11A

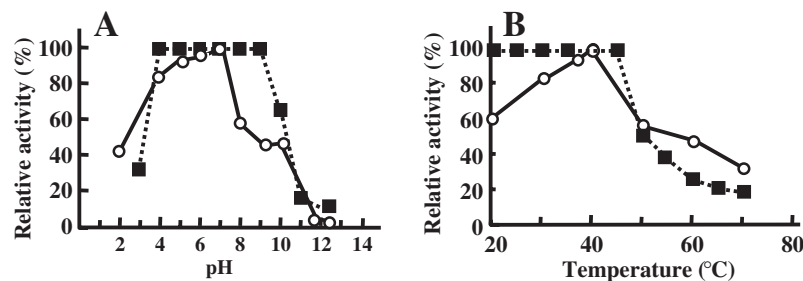
Molecular weight (SDS-PAGE)	about 22 kDa
pH optimum	7
Temperature optimum	40 °C
$K_m$	
Birchwood xylan	9.47 mg/ml
Oat-spelt xylan	13.2 mg/ml
Specific activity	
BWX	628 U <sup>a</sup> /mg of protein
OSX	534 U/mg of protein
PNPX	Not detected
CMC	Not detected
Avicel	Not detected

<sup>a</sup> $\mu$ moles of xylose released per min.

40% in the pH 3 to pH 10 range, even though the pH of maximal activity was around neutral (Fig. 2A). Hydrolytic activity increased at temperatures between 20 °C and 40 °C, but strongly reduced for temperatures higher than 45 °C (Fig. 2B). No significant loss in xylanase activity was observed when the enzyme was stored at 4 °C over a 14-d period. The pattern of xylooligosaccharide, BWX, OSX, and EKP hydrolysis by rXyn11A is depicted in Fig. 3. Xylobiose was the predominant hydrolysis product. Recombinant Xyn11A failed to hydrolyze PNPX, CMC, Avicel (Table 1) or xylobiose (Fig. 3). Divalent heavy metals such as Zn<sup>2+</sup>, Ni<sup>2+</sup>, and Hg<sup>2+</sup> were found to be more than 70% inhibitory when added to activity assay mixtures at a concentration of 1 mM. Mg<sup>2+</sup> and Ca<sup>2+</sup> ions as well as chelating agents (EDTA and EGTA) had no detectable effect on xylanase activity.

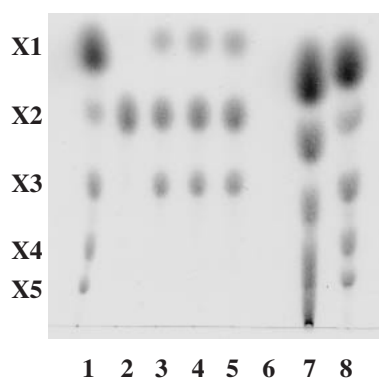
#### Discussion

In terms of yield maximization, the choice of strain background (*i.e.*, specific expression host), vector system, and promoter for heterologous protein expression in *K. lactis* is of particular importance.<sup>2)</sup> For this



**Fig. 2.** Enzymatic Activity and Stability of rXyn11A at Various pHs and Temperatures.

The optimum temperature was found by measuring the enzyme activity with OSX at various temperatures in Britton and Robinson's universal buffer (pH 7.0). The optimum pH was measured by incubating the assay mixtures at different pHs (pH 2.0–12.5) in Britton and Robinson's universal buffer for 30 min at 37 °C. The thermostability and pH stability of the purified enzyme were measured by incubating the enzyme solution at various temperatures (20 to 70 °C) for 1 h in Britton and Robinson's universal buffer (pH 7.0) or incubating the enzyme solution in Britton and Robinson's buffer (pH 2–12.5) overnight at 4 °C, followed by measurement of residual enzyme activities under the standard conditions. A, The effect of pH on activity (open circles) and stability (solid squares). B, The effect of temperature on activity (open circles) and stability (solid squares).



**Fig. 3.** TLC Analysis of Enzyme Hydrolysis Products from Various Xylooligomers and Polysaccharides.

Samples (5–10  $\mu$ l) of each reaction mixture were analyzed by silica gel thin layer chromatography (TLC), using butanol–acetic acid–water (10:5:1) as the solvent system. Reaction products were visualized by spraying a sulfuric acid–ethanol (5:95, vol/vol) solution on the plate, followed by baking at 110 °C for 10 min. Lane 1, standard mixture containing xylose (X1), xylobiose (X2), xylotriose (X3), xylo-tetraose (X4), and xylopentaose (X5); lane 2, X2; lane 3, X3; lane 4, X4, lane 5, X5; lane 6, eucalyptus kraft pulp (EKP); lane 7, birchwood xylan (BWX); lane 8, oat spelt xylan (OSX).

reason, robust combinations of strain, vector, and promoter, as well as new approaches and tools for genetic modification of strain background, are highly sought after. An example is the commercial kit *K. lactis* GG799, which has a track record of excellent protein expression and secretion. In this study, we examined the expression level of a *B. halodurans* recombinant family-11 xylanase in wild-type *K. lactis* CBS 1065 carrying the expression construct pCWK:I-*xyn11A6His*, a derivative of the episomal pKD1-based plasmid pCWK:I, developed by Gibbs *et al.* to overcome the toxic effects of some genes on *E. coli*.<sup>5)</sup> The His-tagged recombinant of *B. halodurans* xylanase Xyn11A was expressed by electrotransformed *K. lactis* CBS 1065 cells at a comparatively high level under the direction of the LAC4 promoter, and efficiently secreted when integrated with the killer toxin secretion signal (Fig. 1A and B). The peak concentration of extracellularly secreted rXyn11A over a 4-d culture period in YPD media using shake flasks was estimated to be 156  $\mu$ g/ml (from the xylanase activity of 98 U/ml in cell free extracellular enzyme preparation and a specific activity of 628 U/mg of purified enzyme against BWX). The extracellular production level for the *B. halodurans* recombinant xylanase was higher than 80  $\mu$ g/ml for *Thermotoga* xylanase XynA<sup>10)</sup> and 130  $\mu$ g/ml for *Dictyoglomus* xylanase XynA<sup>17)</sup> reported for xylanase production in YPD medium by *K. lactis* CBS 1065 carrying a pKD1-based expression vector under shake flask conditions. However, it must be noted that several parameters, including plasmid architecture and type of protein being expressed, can significantly influence xylanase secretion levels for any given expression system.<sup>2)</sup>

*B. halodurans* rXyn11A did not possess detectable cellulase or  $\beta$ -xylosidase activities (Table 1), except for the hydrolysis of xylan substrate. Hydrolysis products were analyzed by TLC, as shown in Fig. 3. The recombinant enzyme hydrolyzed xylo-tetraose and xylo-pentaose to produce mainly xylobiose, with xylo-triose and xylose as minor products. The hydrolytic activity of the enzyme was weak on xylo-triose. It was unable to hydrolyze authentic xylobiose even with prolongation of the incubation time, as has been observed for several xylanases.<sup>14)</sup> When highly polymerized xylans such as BWX and OSX were used as substrates, xylose was detected as a major hydrolysis product, with some xylooligosaccharides as minor products, including xylo-tetraose and xylopentaose. A likely explanation is that a certain degree of polymerization of xylose by the 1,4-linkage is required for the release of xylose monomers. In addition, TLC did not reveal any chain-lengthening transglycosylation reaction products by rXyn11A, even though some alkaliphilic *Bacilli* endoxylanases have been found to have transferase activity.<sup>14)</sup>

A significant industrial utility of some family-11 xylanases is in the biobleaching of kraft pulp.<sup>18–21)</sup> We analyzed sugar (total and reducing) and chromophore (absorbing at 237 and 465 nm) release from EKP by rXyn11A, as described by Elegir *et al.*<sup>22)</sup> and Wood and Bhat,<sup>14)</sup> to validate our TLC result with respect to EKP hydrolysis (Fig. 3). Neither sugars nor chromophores were detectable (data not shown) for EKP, although EKP contained about 10% xylan as a constituent, and rXyn11A reaction mixtures even after a prolonged incubation of 48 h. These findings vouch for a narrow substrate specificity range for *B. halodurans* rXyn11A, as has been observed for some family-11 xylanases, such as *C. stercorarium* xylanase XynA,<sup>23)</sup> *C. cellulovorans* XynA, and *Bacilli* sp. BP-7 endoxylanase.<sup>24)</sup> However, certain rXyn11A properties, such as its broad pH range (pH 3 to pH 10) for activity (Fig. 2A), which are consistent with previous reports on native homologs,<sup>25,26)</sup> are of potential interest in feed and food applications.<sup>27)</sup>

In conclusion, wild-type *K. lactis* CBS 1065 efficiently expressed and secreted *B. halodurans* recombinant Xyn11A. Xylan hydrolysis by recombinant xylanase contributed mainly to the depolymerization of  $\beta$ -1,4-linked chains in xylan. Neither sugars nor chromophores were detectable from EKP, indicating that different family 11 xylanases might not be equivalent with respect to their action against kraft pulp. Even though *B. halodurans* recombinant Xyn11A had a narrow substrate specificity range, other properties, such as pH stability, make it a worthwhile candidate for improvement and/or cloning in industrial yeast strains. Heterologous expression systems that produce large amounts of secreted proteins with an organism that can be grown in industrial scale fermenters must be developed to facilitate enzyme production. Since optimization of growth medium is a useful tool to attain high levels of enzyme

activity at a lower cost,<sup>28)</sup> further studies on medium optimization might improve the yield of *B. halodurans* recombinant Xyn11A with the kind of *K. lactis* expression system described in this paper. An important future consideration is to develop higher-level enzyme producing integrant *K. lactis* strains with a number of copies of Xyn11A for growth in optimized media.

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