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The effective expression of xylanase gene in *Candida utilis* by 18S rDNA targeted homologous recombination in pGLR9K

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Abstract In order to test whether 18S rDNA can influence positively xylanase gene effective expression in the yeast of *Candida utilis*, a targeting vector pGLR9K-XA was constructed by adding an interested gene *xyn*A from *Streptomyces olivaceoviridis* into the vector pGLR9K which is constructed by ourselves. pGLR9K contains the 18S rDNA, GAP promoter and CYH resistance gene sequence, all of which is from *C. utilis*. Then the vector pGLR9K-XA was transformed into *C. utilis*. To test the vector and transformed system, PCR, Southern blot and DNS methods were used. The results showed that xylanase gene can be detected in the chromosome DNA of recombinant *C. utilis* and the enzyme activity of xylanase is up to 60 IU ml^{-1} in the study. It is suggested that this system can be used to express exogenous genes in *C. utilis* as a bioreactors. This is the first report that xylanase gene was expressed in C. *utilis*.

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Introduction

Escherichia coli is a usual expression system, but it is a non-food bacterium and we can not use it in food. However, *Candida utilis*, as a biosafety microorganism, is an important food yeast, which can be fermented in high cell density liquid, And does not need be induced by ethanol or methanol compared with *Pichia*. In our previous research, The *C. utilis* was added to the silage with other silage microorganisms. The mixed microorganisms has been proved very effectively and applied in silage yet in our province [1]. So we have proposed the hypothesis that use the *C. utilis* to produce xynase in silage, which will hydrolyze xylan into xylose for improving the silage quality more.

There are some reports on genetic engineering in *C. utilis*. And there has some considerable progress in the area in recent years. Because of the feasibility of expression of exogenous genes in yeast, The *C. utilis* that is regarded as high biological safety yeast is also expected to be a bioreactor to produce xylanase. Some exogenous genes have been accomplished in *C. utilis* [2], such as foreign Lycopene, Carotene, Carotenoid and Astaxanthin expressed in *C. utilis* [3, 4]. The *C. utilis* is a member of silage inoculant. Silage is one of the largest agricultural processes in China. But there has been a great loss in silage due to the non-hydrolyzed cellulose and hemicellulose, so there has been considerable effort to improve silage quality via genetic transformation technology.

The reliable and stable expression of transgenesis is a prerequisite for the successful application of gene

technology. However, the expression of transgenes is generally low compared to that of the corresponding endogenous genes and transgene silencing may even occur. Homologous recombination (HR) technology offers an alternative to settle this difficulty [5]. HR permits the precise insertion of foreign genes to specific sites in chromosome of host cells. The rRNA gene sequence can be used as homologous sequences to direct HR, whose sequence is highly conserved and exists in multiple copies in organisms. The rRNA gene sequence as a HR sequence has been tried in yeast [6], rice [7], Arabidopsis thaliana [8] and a bacterium [9], and the expression of exogenous genes was highly improved in these experiments. The multiple target sites of HR provide more integration chance and the insertion inactivation of at least some copies is neither lethal, nor slowly the cell growth rate [10].

At present, the strong promoter and terminator sequences of the glyceraldehyde-3-phosphate dehydrogenase (GAP) gene are used for achieving high and stable expression of the target genes. This technology has been successfully applied in yeast [11, 12].

Xylanase can hydrolyze xylan which belongs to hemicellulose, and its product of hydrolyzation is very important in food and other industries. Because of that, we chose xylanase A (*xyn*A) as a target gene, which is the most important and the most attention gene in xylanase gene family.

Because the *C. utilis* is a kind of food yeast, it can not be substituted in some industries. For example, their product can be as an additive without purification in food and other industries, which is perfect to that of *E. coli* and *Pichia pastoris*. So we design the experiment to settle for our research need. The vector of the study reported here was to use 18S rDNA as a HR gene; the GAP gene promoter as a strong promoter; mL41 gene as a selectable marker gene, which is resistant to cycloheximide (CYH); and xylanase as a target gene to evaluate the effect of HR on the expression of xylanase gene in *C. utilis*.

Materials and methods

Preparation of chromosomal DNA of C. utilis [13]

The strain of *C. utilis* was inoculated into 20 ml YPD medium and cultured at 30°C to early stationary phase. The cells were harvested by centrifugation at 9,000×*g*, suspended in extraction buffer (10% SDS, 50 mM EDTA, 50 mM Tris–HCl, pH 8.0, 3 μl 20 mg/ml protease K) and incubated in a water bath at 37°C for 1 h. 5 M NaCl and cetyltriethylammnonium bromide (CTAB) was added and the mixture was incubated at 65°C for 30 min. After extracting with equal volumes of phenol: chloroform (1:1), the total chromosomal DNA was precipitated with 1/10 volume of 3 M sodium acetate and two volumes of ethanol. The pellet was resuspended in TE buffer with RNase and stored at −20°C.

PCR amplification and cloning

The primers F-1 (5′CGATATCTGCCAGTAGTCATA TGC3′) and R-1 (5′CGATATCTGACTTGCGCTTACT AG3′) were designed for amplification of 18S rDNA (AF239662). Each primer contains an upstream *Eco*RV site. Amplification was performed as follows: 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 57°C, 2 min at 72°C; followed by final extension for 10 min at 72°C. The resulting 1.7 kb PCR product of 18S rDNA was cloned to the T-vector (TaKaRa) (Fig. 2a), digested by *Eco*RV, and sub-cloned to the vector pGL (W Wang, XAAS) and transformed into *E. coli* strain *DH5a*. The resultant recombinant plasmid was verified by PCR and restriction enzyme analysis. The plasmid was named as pGLR9K (Fig. 1).

Construction of expression vector pGLR9K-XA

The primers F-2 (5′TATCTAGAATGGCCGAGAGCACG CT3′) and R-2 (5′GGTACCTCAGGTGCGGGTCC3′) were used to amplify the complete *xyn*A open reading frame (1.3 kb) from genomic DNA of *Streptomyces olivaceoviridis*. F-2 contains an upstream *Xba*I site, and R-2 contains an upstream *Kpn*I site. The xynA ORF does not contain a predicted secretion signal peptide.

The expression vector pGLR9K-XA was constructed by inserting the xylanase gene (*xyn*A) into the vector pGLR9K, which contains the GAP promoter, *xyn*A,GAP

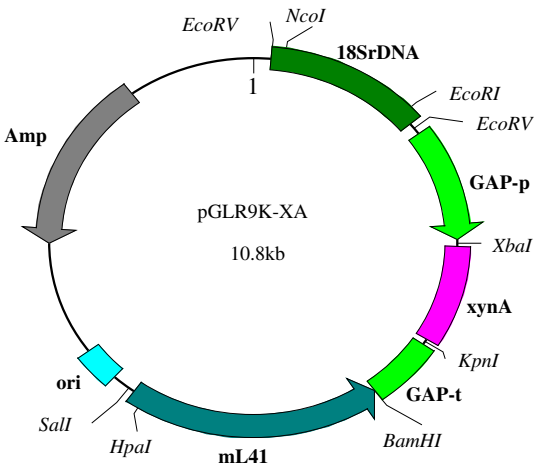

Fig. 1 The structure of vector pGLR9K-XA: *xynA* was inserted into pGLR9k, which is constructed by ourselves through adding GAP-p, GAP-t, mL41 and 18S rDNA to pBR322. *NcoI, HpaI* and *EcoRI* are linearing sites

terminator, mL41 and 18S rDNA gene sequence (Fig. 1). The recombinant plasmid pGLR9K-XA was verified by restriction enzymes analyses and sequencing. The plasmid DNA extraction and purification was carried out consulting the Molecular Cloning: A Laboratory Manual, 3rd ed [13].

Transformation

Electroporation was used for transformation. The cells of *C. utilis* were cultured overnight in YPD, inoculated to fresh medium and grown to a logarithmic phase (OD_{600}: 1.5–2.0). Cells were harvested by centrifugation at 6,000×*g*. The pellet was washed twice with ice-cold sterile water and once with 1 M sorbitol, then diluted to 10^6 cells per ml with 1 M sorbitol. The resulting *C. utilis* cell suspension was mixed with 0.1 μg plasmid pGLR9K-XA linearized with restriction enzyme *Nco*I, *Hpa*I or *Eco*RI (TaKaRa) and electroporated utilizing a 0.2 cm curvette. *C. utilis* cell suspension mixed with plasmid-free sterile water was used as negative control. Per standard protocol, the electric pulse voltage was set at 1,000 V with a pulse length of 5×10^{-3} s (BIO-RAD Micropulser). After electroporation, 1 ml of ice-cold YPD medium containing 1 M sorbitol solution (YPDS) was immediately added to the reaction cuvette, gently mixed and subsequently cultured for 5 h at 30°C. The mixture was spread on YPD plate containing 50 μg/ml cycloheximide and incubated at 30°C until colonies appeared.

PCR analysis of foreign genes incorporation

To confirm integration of heterogenous genes in the genome, PCR analysis was performed. Genomic DNA was extracted from transformants of *C. utilis*. Primers F-2 and R-2 for amplification of 1.3 kb fragments of xylanase gene were designed to test the incorporation of xylanase gene in genome of *C. utilis*. Amplification was performed as follows: 5 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 58°C, 1.5 min at 72°C; followed by final extension for 10 min at 72°C. The PCR product was analyzed by agarose gels electrophoresis.

Analysis of xylanase activity

Xylanase activity in recombinant *C. utilis* was measured using the modified 3, 5-dinitrosalicyclic acid (DNS) method [14, 15]. Oat spelt xylan (Sigma) was used as a substrate in the analysis. Recombinant yeast and the fermented product were harvested by centrifuged after fermentation for 72 h and washed the cells once with xylanase extraction buffer (Disodium hydrogen phosphate–citric acid buffer, pH5.6). Then resuspended it with 0.5 ml extraction buffer. Ultrasonic wave was used to break recombinant yeast cell walls. The samples were centrifuged at 4°C (10,000×*g*) for 5 min. A 0.2 ml supernatant was incubated for 10 min at 55°C with 1.8 ml substrate whose concentration is 0.5% (w/v). To stop the reaction, the 3 ml DNS was added. After boiled for 7 min, the mixture was cooled with cold water at once. Then the product was measured with a spectrometer (UV-2550, DAOJIN UV–Visible-spectrophotometer. Japan) at 540 nm. Xylanase specific activity was calculated by using xylose as standard. When producing 1 μmol reducing sugar, the amount of exhausted xylanse in one minute is defined as a xylanase activity unit (IU).

Analysis of southern blot

Extract DNA from *C. utilis* transformed and non-transformed. Electrophorese it in 0.8% agarose. Then transfer the agarose into nylon membrane (Roche, Mannheim, Germany). PCR DIG Probe Synthesis Kit (Roche) labels the 1.2 kb xylanase gene from PCR. The operation of southern blot follows the DIG High Prime Labeling and Detection Starter Kit I (Roche).

Results

PCR identification

The 1.3 kb product was amplified from genome of transformed *C. utilis* protoplasts with primers F-2 and R-2 (Fig. 2b). Compared with yeasts transformed with plasmid-free sterile water, only yeasts transformed with pGLR9K-XA present a 1.3 kb product. It suggests that the exogenous xylanase gene has been incorporated into the genome of *C. utilis*. These results prove that HR occurred in *C. utilis* transformed with pGLR9K-XA.

Identification of xylanase activity

The xylanase activity was quantitated with DNS analysis after hydrolyzing oat spelt xylan into xylose. Compared with yeasts transformed with plasmid-free sterile water, only yeasts transformed with pGLR9K-XA exhibited a tremendous specific xylanase activity in our assays. In our experiment, the specific xylanase activity was greatly affected by the linearing site of recombination plasmid. Compared with plasmid linearized with *Nco*I restriction enzyme, the activity of xylanase was higher when linearized with *Hpa*I or *Eco*RI, but the activity of xylanase is the highest when using *Eco*RI restriction enzyme to linearize vector (Fig. 3). Yeast cell transformed by *Eco*RI-linearized vector showed higher xylanase activity, which maybe that because *Eco*RI is located on the downstream of the code

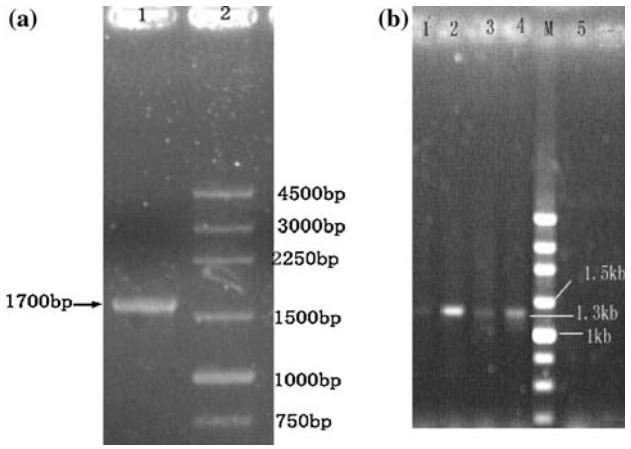

Fig. 2 **a** The PCR production of 18S rDNA: (PCR amplification by using primers F-1 and R-1): lane *1*, The PCR production of 18S rDNA; lane *2*, 250 bp Marker. **b** PCR Identification of xylanase gene incorporation (PCR amplification by using primers F-2 and R-2): lane *1*; lane *2*; lane *3*; lane *4*, yeasts transformed with pGLR9K-XA; lane M, 250 bp Marker; lane *5*, yeasts transformed with plasmid-free sterile water as a control. There is no band in lane *5*. Lane *1*, lane *2*, lane *3* and lane *4* are different yeast transformants

chain, it will not restrain the transcription of 18S rDNA. On the opposite, *Nco*I is locus on upstream and will restrain the transcription. Therefore, the *Nco*I is not the excellent site as a linearing site. The activity of xylanase was about 60 IU ml^{-1} in our experiment. The xylanase activity in both intracellular and extracellular part of transformed *C. utilis* can be detected (the data of the extracellular xylanase activity is not shown there), the cause of which may be that the broken cell wall leaks xylanase from intracellular to extracellular part following the fermentable metabolize or other reasons. Yeasts transformed exhibited an intense CYH resistance (about CYH concentration 100 μg/ml), whereas the yeasts transformed with no plasmids exhibited no CYH resistance. This result is consensus with the plasmid incorporated to the chromosome of *C. utilis.* Yeast cell transformed by *Hpa*I-linearized vector could exhibit CYH resistance. We think the reason for it is that *Hpa*I is not in the middle of code region, the ORF of mL41 can express CYH resistance protein smoothly.

Southern blot analysis

Southern blot showed whether the xylanase gene is integrated into the genomic DNA of *C. utilis*. The genomic DNA of *C. utilis* without being reconstructed is as a control. The genomic DNA of *C. utilis* transformed with xylanase gene which showed transformed successfully in PCR test is as tested materials. The 1.2 kb xylanase gene was labeled as probe to hybridize the genomic DNA. The hybridization in recombinants presented effective band, however, in original *C. utilis* it presented no band (Fig. 4). The result shows xylanase gene integrated into the genomic DNA of *C. utilis*.

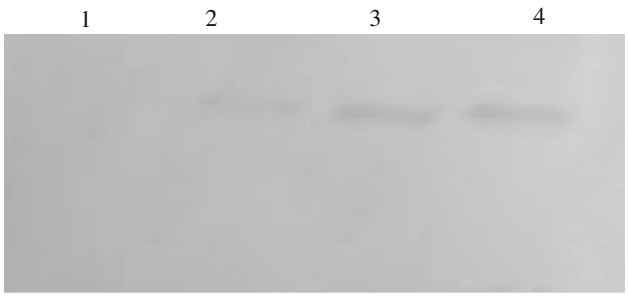

Fig. 4 Southern blot of genomic DNA from transgenic *C. utilis*: *Lane 1* showed the genomic DNA of *C. utilis*; *Lane 2–4* showed the recombinant genomic DNA of *C. utilis* containing xylanase gene, which generated from *Eco*RI-linearized vector

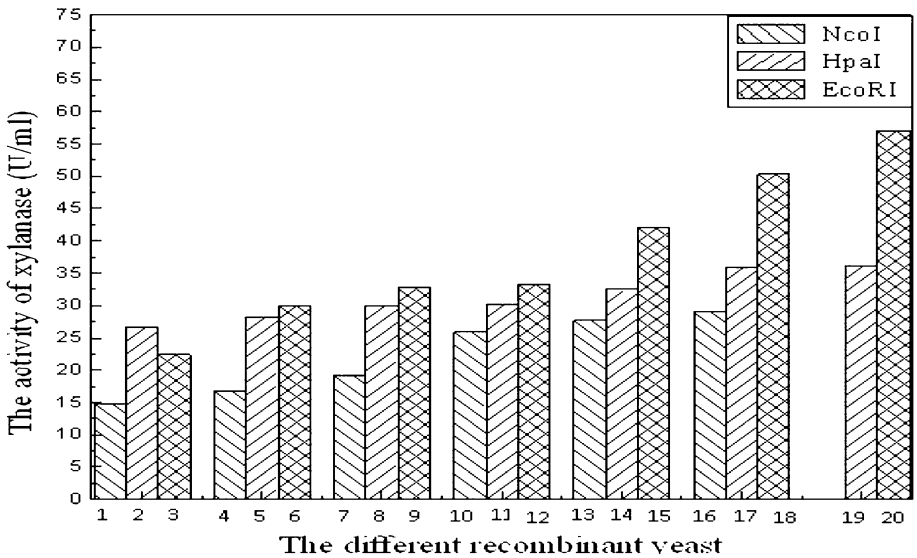

Fig. 3 The comparison of xylanase activity among three sites linear transformation. The xylanase activity of the yeast without xynA is zero as a control, so it is not shown. The *x*-axis indicates different yeast clones transformed

Discussion

The *C. utilis* is an important industrial microorganism. It has been established by the Food and Administration (FDA) to be of high biological safety. So it can be used to produce useful substances with recombinant DNA technology [16]. As a good receptor for transgenic assay, it has some advantages: firstly, *C. utilis* can generally be cultured at a cell higher density than that of the other yeasts. Secondly, it does not produce ethanol under aerobic conditions [17]. The most important, some reports show it can express exocrine enzyme. Some research workers deduced that because it can metabolize pentoses and xylose [18], *C. utilis* can express related enzymes from heterogeneity gene. In this study, the heterogeneous xylanase can be expressed successfully in *C. utilis*. It suggests that the *C. utilis* with pGLR9K can be an effective expression system.

Xylan is a major component of hemicelluloses in plant cell walls. Xylan and their hydrolysates are useful substances, with importance in both animal feed and paper industries [19]. In our study, recombinant xylanase from *C. utilis* transformant can hydrolyze xylan into xylo-oligosaccharides of varying lengths. Thus, the system that *C. utilis* express heterogeneity xylanase can be used in silage, which is a considerable project. The electroporation method was used to transform due to its simplicity and successful application in *C. utilis* [20, 21].

The 18S rDNA unit of *C. utilis* was cloned and used as a guider to integrate the vector DNA into the *C. utilis* chromosome. It exists as multiple repeated copies in the genome of *C. utilis*. Integration of vector DNA at the rDNA locus has been shown to result in a higher transformation efficiency compared with that single-copy gene [17, 22]. The southern blot analysis shows also *xyn*A integrated into the genomic DNA of *C. utilis*. Our conclusion is unanimous with Y. R. Hong [2].

Plasmids of pGLR9K containing the 18S rDNA fragments have been shown to transform yeast successfully after linearization, and the efficiency of recombination is affected by the site linearized in the HR sequence. In our study, the HR expression vector pGLR9K-XA was designed as a replacement vector to give a region of homology to the chromosomal locating on both of its ends. It locates the exogenous DNA on the 18S rDNA of *C. utilis* with recombination. Some reports show that the suitable length of homologous fragment was between 1 and 2 kb considering that shorter targeting fragment would decrease the efficiency of recombination, while longer targeting fragment would increase the difficulty of operation [23, 24]. In the study, the length of the homologous fragments is 1.7 kb. To our surprise, the transformed yeast exhibits xylanase activity when transformed with *Hpa*I-linearized plasmid vector, the homologous sequence of which is in mL41. Yeast cell transformed by *Hpa*I-linearized vector could exhibit xylanase activity. The reason of that, we think, is that mL41 is homological with L41. Between those only the sequence for one amino acid is different, it can also be as HR sequence except the low HR efficiency. In this study, we have accomplished the expression of foreign genes in *C. utilis* so far. However, the *Eco*RI-linearized maybe not the most efficient site in 18S rDNA. For further study, the research on the function of the HR vector with 18S rDNA as homologous fragment, the choice of the optimization vector and steady expression experiments of foreign genes are needed. Such work is under way in our laboratory.

In summary, the results presented above demonstrate that the HR vector pGLR9K, which uses the multicopy and conserved sequences 18S rDNA as a guider, can express xylanase gene in *C. utilis*. This system will can be applied in our silage experiment. Thus, it is possible that this technique can be used to express other heterogenous genes in *C. utilis* with vector pGLR9K as a medium after the vector optimized. The strategy described here also makes it possible to explore the potential of *C. utilis* as a bioreactor to produce useful foreign protein.

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