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Overexpression of both the lactase gene and its transcriptional activator gene greatly enhances lactase production by *Kluyveromyces marxianus*

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ABSTRACT

After both a lactase gene *(LAC4)* and its transcriptional activator gene (*LAC9*) were expressed in *Kluyveromyces marxianus* KM-69 which was a glucose-derepressed mutant, one transformant called KM-L9-20 grown in an optimized whey medium for lactase production could produce 77.8 ± 4.0 U/ml of lactase activity at a flask level within 36 h. At the same time, expression of the *LAC4* gene and its transcriptional activator gene in the transformant KM-L9-20 were also greatly enhanced compared to that of the *LAC4* gene and the *LAC9* gene in its parent strain *K. marxianus* KM-69. 157.9 ± 3.2 U/ml of lactase activity was yielded by the transformant KM-L9-20 within 84 h during a batch fermentation while 274.7 ± 4.3 U/ml of lactase activity was produced within 48 h during a fed-batch fermentation. The produced lactase could actively transform lactose into the galacto-oligosaccharides which contained mainly trisaccharides. The optimal conditions for the transgalactosylation reaction were that the lactose concentration, the reaction temperature, pH and the lactase dose were 400 g/l of lactose, 40 °C, 6.5 and 25 U/g of lactose, respectively. Therefore, the transformant KM-L9-20 and the produced lactase had highly potential applications in biotechnology.

1. Introduction

In recent years, *Kluyveromyces marxianus* and the produced lactase and inulinase by it have been receiving great interest in biotechnology [1,2]. This is due to the fact that *K. marxianus* has the high capacity to assimilate lactose and inulin, short generation time, and a high secretory capacity, can tolerate high temperature, and its growth rate and growth yield are extremely rapid and high [3]. The yeast also has an acceptability as a safe microorganism, meaning that it can be safely used in the industrial biotechnology [3]. Lactase, also called β-galactosidase catalyzes hydrolysis of lactose in milk and whey, producing a mixture of glucose and galactose [4]. Most β-galactosidases are produced by *Aspergillus niger*, *Aspergillus* oryzae, *Kluyveromyces lactis* and *K. marxianus*, because they can have acceptable productivities and yields of lactase [5]. A lactase in *Kluyveromyces* spp. is intracellular; lactose is first transported to the interior of the yeast cell by a permease and then hydrolyzed intracellularly to glucose and galactose [5,6]. The lactase produced by *K. marxianus* has been purified and characterized [7] and a gene encoding the lactase in this yeast has been cloned and characterized [8]. It was found that ORF (accession number: AY526090.1) of the gene has 3078 bp long. If the copy number of the lactase gene in this yeast can be increased, lactase production in the engineered yeast will be enhanced. It has been well known that the gene expression is repressed by high concentration of glucose and greatly induced by lactose and galactose available in the medium [1,9]. It has been confirmed that in the presence of an inducer (lactose) a transcriptional activator that contains an amino acid sequence of the form Cys-X2-Cys-X6- Cys-X5-12-Cys-X2-Cys-X6-8-Cys can bind to the consensus sequence 5′-CGG(N5)A/T(N5)CCG-3′ in the promoter of the lactase gene and other genes encoding galactokinase, galactose-1-phosphate uridylyltransferase, and UDP-glucose-4-epimerase and a lactose permease in *K. lactis* to induce their expression (Fig. 1) [10]. Therefore, after a gene encoding a Mig1, the transcriptional repressor was removed in *K. marxianus*, lactase and inulinase production by the disruptants was significantly improved [1,2]. However, it is still unknown whether or not over-production of the transcriptional repressor in this yeast cells can enhance expression of the lactase gene and promote lactase production.

The lactase produced can be used for hydrolysis and removal of lactose of milk in dairy industry and of whey in environment, for prevention of lactose crystallization and increase its sweetening power in food industry, for treatment for alleviating symptoms of lactose

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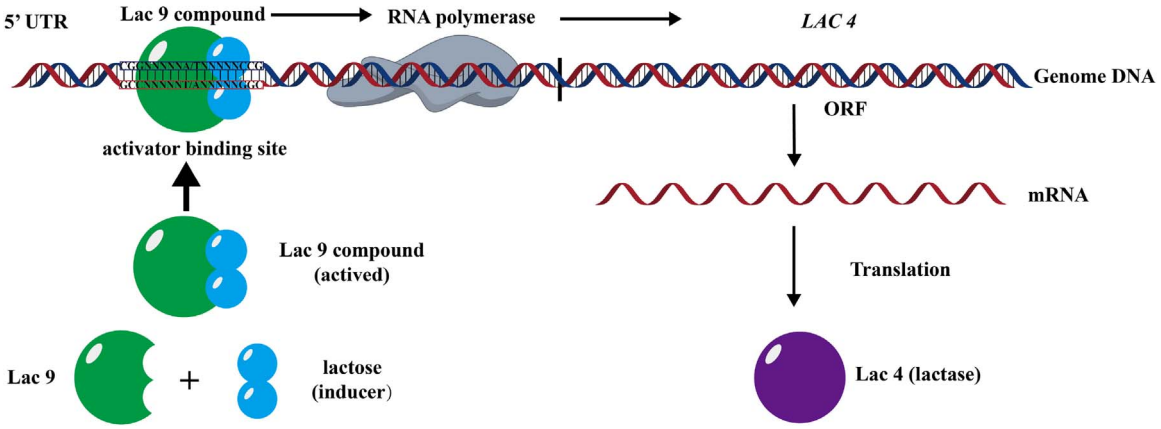

Fig. 1. The proposed role of the transcriptional activator during the induction by lactose.

intolerance in human. It also can be utilized for production of ethanol, galactooligosaccharides and galactose. Therefore, it is meaningful how to enhance lactase production by this yeast through molecular engineering. In recent years, the main focus has been on the expression and production of *Aspergillus niger*, *Kluyveromyces lactis* and bacterial β-galactosidases in different microbial hosts [6]. However, although heterologous expression of the *K. lactis* β-galactosidase gene was carried out by many researchers [6], little has been done on homologous expression of the *K. marxianus* β-galactosidase gene. Therefore, in this study, both the lactase gene and its transcriptional activator gene were overexpressed in a *mig1* mutant KM-69 (glucose derepressed mutant) of *K. marxianus* and a transformant overexpressing both the lactase gene and its transcriptional activator gene was used for over-production of lactase.

2. Materials and methods

2.1. The strains, media and plasmids

A disruptant KM-69, a glucose-derepressed mutant, was obtained by disruption of the *MIG1* gene in *K. marxianus* KM-0, which is being widely used for lactase production on a large scale in China [1,11]. The cultivation medium for the yeast growth was a YPD medium containing 20.0 g/l of glucose, 20.0 g/l of polypeptone and 10.0 g/l of yeast extract. A whey medium for lactase production was as follows: 120 g/l of whey powder, 10.0 g/l of polypeptone, 15.0 g/l of yeast extract, 1.0 mM of $MnCl_2$, pH 7.0. The *Escherichia coli* strain used in this study was DH5α [F^- *endA1 hsdR17* ($rK\_/mK^+$) *supE44 thi*$^-$ λ$^-$*recA1gyr96DlacU169* (*j80lacZDM15*)] and was grown in 5.0 ml of a Luria broth (LB) at 37 °C overnight [12]. A plasmid pPWN302 carrying the gene encoding the nourseothricin resistance and an overexpression vector pMD-rDNA-G418 in *K. marxianus* KM-0 were constructed in this laboratory. A plasmid pMD19-T simple for cloning of PCR products was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China).

2.2. Whey powder

The whey powder (lactose content was 77 g/l) used in this study was purchased from Jinan Biotechnology Company, China.

2.3. Construction of the expression vectors for overexpression of the homologous lactase gene (LAC4 gene) and the transcriptional activator gene (LAC9 gene)

The ORF and the terminator of the *LAC4* gene were PCR amplified from the genomic DNA of the *mig1* mutant KM-69 using the primers LAC4-F and LAC4-R designed based on the accession number AY526090.1 and the promoter of the *LAC4* gene was PCR amplified from the genomic DNA of the same *mig1* mutant KM-69 using the primers LAC4-UP-F and LAC4-UP-R designed according to the accession number AP014601.1 (Table 1). After the DNAs were sequenced and the two fragments were spliced, a full length (4152 bp) of the *LAC4* gene in the *mig1* mutant KM-69 was obtained. In order to enhance expression of the *LAC4* gene and lactase production, first, the expression vector for overexpression of the homologous *LAC4* gene was constructed (Fig. 2). Based on the plasmid pMD-rDNA-G418 constructed in this laboratory [2], the G418 resistance gene in the pMD-rDNA-G418 was replaced by the gene encoding the nourseothricin resistance from the plasmid pPWN302, forming the plasmid KMNAT1 (Fig. 2). Then, the ORF and the terminator of the *LAC4* gene as well as the promoter of the *LAC4* gene were ligated into the plasmid KMNAT1, resulting in the plasmid KMNAT1-LAC4 (Fig. 2).

In order to simultaneously over-express the transcriptional activator

Table 1
The primers used in this study.

Primer	sequence
LAC4-UP-F	5′-GAGCTCATTCCCATTTGGAGTAGGAAA-3′ (The underlined bases encode *Sac*I site)
LAC4-UP-R	5′-GTCGACATCTTTCAGTTCTCGATGAGTATG-3′ (The underlined bases encode *Sal*I site
LAC4-F	5′-GTCGACATGTCTTGCCTTATTCCTGAG-3′ (The underlined bases encode *Sal*I site)
LAC4-R	5′-ACTAGTCTGCAATTGTTTCTAAGCATGCTG-3′ (The underlined bases encode *Spe*I site)
KM-ACT-F	5′-CAGAGGTCGCTGCTTTAGTTATTG-3′
KM-ACT-R	5′-ACCCATACCGACCATAATACCTTG-3′
L4-YG-F	5′-CGATTGAGTCGTTCGAGCACA-3′
L4-YG-R	5′-GACCACTAAGTTTTCGCCCTCAG-3′
LAC9-P-F	5′-GTCGACACAGCACAAAAAAAGCCCAGA-3′ (The underlined bases encode *Sal*I site)
LAC9-P-R	5′-AGATCTATGTAAAACCTTCCCTATTCCACC-3′ (The underlined bases encode *Bgl*II site)
LAC9-CDS +T-F	5′-AGATCTATGCGGAAATATAAGGTGGTG-3′ (The underlined bases encode *Bgl*II site)
LAC9-CDS +T-R	5′-CTGCAGAAAGATGAGTTTAGTGATGGGA-3′ (The underlined bases encode *Pst*I site)
NAT1-F	5′-AACCAACAACTAGAAATGACCACTTTGGACG-3′
NAT1-R	5′-CTAGAACCTAATTTAATTTATGGACATGGCATGG-3′
G418-F	5′-ATGGGTAAGGAAAAGA-3′
G418-R	5′-TTAGAAAAACTCATCGAGC-3′
L9-YG-F	5′-GTCATGGGGAACTTCTCTGGCT-3′
L9-YG-R	5′-CAGTGTCTGATGGAGTCCTTGCT-3′