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Enhanced production of extracellular inulinase by the yeast *Kluyveromyces marxianus* in xylose catabolic state

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The production of extracellular proteins by the thermotolerant yeast *Kluyveromyces marxianus*, which utilizes various sugars, was investigated using media containing sugars such as glucose, galactose, and xylose. SDS-PAGE analysis of culture supernatants revealed abundant production of an extracellular protein when cells were grown in xylose medium. The N-terminal sequence of the extracellular protein was identical to a part of the inulinase encoded by *INU1* in the genome. Inulinase is an enzyme hydrolyzing β-2,1-fructosyl bond in inulin and sucrose and is not required for xylose assimilation. Disruption of *INU1* in the strain DMKU 3–1042 lost the production of the extracellular protein and resulted in growth defect in sucrose and inulin media, indicating that the extracellular protein was inulinase (sucrase). In addition, six *K. marxianus* strains among the 16 strains that were analyzed produced more inulinase in xylose medium than in glucose medium. However, expression analysis indicated that the *INU1* promoter activity was lower in the xylose medium than in the glucose medium, suggesting that enhanced production of inulinase is controlled in a post-transcriptional manner. The production of inulinase was also higher in cultures with more agitation, suggesting that oxygen supply affects the production of inulinase. Taken together, these results suggest that both xylose and oxygen supply shift cellular metabolism to enhance the production of extracellular inulinase.

[**Key words:** Inulinase; Xylose; *Kluyveromyces marxianus*; Extracellular production; Aerobic catabolism]

Microorganisms produce various types of extracellular sugar-hydrolyzing enzymes to utilize polysaccharides such as starch and cellulose. The production of these extracellular enzymes are often induced or enhanced by sugars and chemicals which are similar to the target polysaccharides. For example, synthesis of extracellular endo-1,4-β-xylanase in the yeast *Cryptococcus albidus* is induced by xylan and xylooligosaccharides to a great extent (1). The production of extracellular glucoamylase in the yeast *Schwanniomyces castellii* is induced by maltose at the transcriptional level (2).

Inulin is a polysaccharide synthesized and stored in tubers and roots of many plants, including the Jerusalem artichoke and chicory (3). Structurally, inulin is composed of fructose joined by β-2,1-fructosyl bonds with a glucose at the reducing end. Inulinase is an enzyme that degrades the β-2,1-fructosyl bond to produce fructose from inulin. The production of fructose is a target of biotechnological industries because fructose is sweeter than sucrose and has several positive effects in foods, beverages, and even pharmaceuticals (4). Currently, fructose is produced by hydrolysis of starch followed by isomerization of the liberated glucose. The isomerization efficiency following starch hydrolysis is at most 50%; however, inulinase has the potential to produce higher concentrations of fructose syrup from inulin (5).

The yeast *Kluyveromyces marxianus* is a thermotolerant yeast that can grow at temperatures up to 50°C (6,7). *K. marxianus* produces ethanol at higher temperatures than the conventional ethanol-producing yeast *Saccharomyces cerevisiae* (8). Therefore, *K. marxianus* has the potential of establishing a high-temperature fermentation process that is effective in reducing the cost of bioethanol production (9). In addition, *K. marxianus* has recently received attention as an efficient producer of inulinase for the purposes of producing fructose from inulin (10,11).

Many microorganisms, including *K. marxianus*, have been screened as inulinase producers. Several yeasts as well as many filamentous fungi, such as *Aspergillus* spp. and *Rhizopus* spp., are known to produce inulinase (12). In screening experiments, yeast strains were cultured in a medium containing inulin (13). An *Aspergillus tubingensis* strain was tested for the production of inulinase in media containing plant biomass or pure sugar. Chicory root powder, known to contain inulin, was the best substrate, and glucose was the worst carbon source for the production of inulinase (14). In the case of the *K. marxianus* YS-1 strain, inulin was also the best carbon source among the sugars tested, including inulin, fructose, glucose, lactose, maltose, starch, and sucrose, for the production of inulinase (15). The *K. marxianus* strain CBS 6556

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produced more inulinase using sucrose than using fructose, glucose, glycerol, or ethanol in chemostat cultures (16). The production of inulinase has been generally studied in culture media containing pure inulin or inulin-containing biomass (12).

In addition to the sugars used in the aforementioned studies on *K. marxianus* strains YS-1 and CBS 6556 (15,16), *K. marxianus* has been shown to grow well by consuming cellobiose, xylose, xylitol, or arabinose as a sole carbon source (8), a useful feature for the production of various biological materials. In this study, we examined the production of extracellular proteins in media containing various sugars utilized by *K. marxianus*. We found that xylose specifically enhanced the production of extracellular inulinase, encoded by *INU1* though the Inu1 protein was not required for xylose utilization. Further study suggested that xylose consumption and aerobic metabolism are suitable for the production of extracellular inulinase by *K. marxianus*.

MATERIALS AND METHODS

Strains and media Strains used in this study were obtained from the National Collection of Yeast Cultures (NCYC, UK) and the Biological Resource Center, NITE (NBRC, Japan). The strain DMKU 3−1042 is identical to NBRC104275. Yeast strains were cultured in media containing 2% polypeptone, 1% yeast extract, and 2% sugar (glucose (YPD), sucrose, xylose (YPX), xylitol, galactose, arabinose, lactose, or cellobiose). For growth measurements of the *Δinu1* strain, filter-sterilized minimal medium (0.17% Yeast Nitrogen Base without amino acid and ammonium sulfate (US Biological, Salem, MA, USA), 0.5% ammonium sulfate) containing 2% sucrose or inulin as a sole carbon source was used.

Culture and sample preparation for analysis For small-scale cultures, two-mL of media was used in test tubes and one-mL was used in 24-well plates. Yeast cells were inoculated and cultured at 30°C with agitation at 50 rpm/min rotation (Rotator RT-50, Taitec, Koshigaya, Japan) for test tubes and at 150 rpm rotary shaking (Double shaker NR-3, Taitec) for plates. To analyze the effect of dissolved oxygen (DO) on the production of inulinase, jar fermenters (250 mL, Mitsuwa Frontech, Osaka, Japan) were used. Yeast cells were inoculated into 200 mL YPX and cultured at 30°C with 1 vvm aeration and 400−1200 rpm agitation. DO and pH were measured using InPro6800/12/120 (Mettler Toledo, Columbus, OH, USA) and InPro3030/120 (Mettler Toledo), respectively. An aliquot of cultures was appropriately diluted for measuring cell density as OD at 600 nm. For analyzing extracellular protein production, inulinase activity, and sugar concentration, cultures were centrifuged and the resulting culture supernatants were used.

To measure growth of the Δ*inu1* strain, cells were grown at 30°C with 70 rpm agitation. OD at 660 nm was automatically monitored every 15 min using a Biophotorecorder (TVS062CA, Advantec, Tokyo, Japan).

Strain construction For *INU1* deletion, a DNA fragment was prepared by PCR with 60 bp homologous sequences at the ends to delete the entire *INU1* coding sequence. The DNA fragment was used for transforming a *Δku70* strain of *K. marxianus* using the method previously reported (17). Accurate disruption was confirmed by PCR.

For reporter assays, the *INU1* and *ACT1* promoters were cloned into a plasmid to express yNLuc (18) using functional marker selection (19). The *INU1* promoter (*INU1*p) and *ACT1* promoter (*ACT1*p) in *K. marxianus* were amplified by PCR using the primers URA3+772786TGA-KmINU1-1487 (5′-gcatatttgagaagaTGAAAATCCGTCCGCCGTAGGTTGACTG-3′) and KmINU1-1c (5′-ATCTAACAAAAAAAAAATTAAATGTGTCAC-3′) for *INU1*p and URA3+772786TGA-KmACT1-1644 (5′-gcatatttgagaagaTGATCGCGGTACAACGTATTAACTCCTG-3′) and KmACT1-1c (5′-AGAACACTAACACGTAAACAGCCCA-3′) for *ACT1*p with chromosomal DNA of the DMKU 3−1042 strain as a template. Vector DNA was also amplified by PCR using the primers URA3+771c (5′-TTCCCAGCCTGCTTTTCTGTAACGT-3′) and yNLuc+1 (5′-ATGGTCTTCACCTTGGAAGACTTCG-3′) with a plasmid (YCp11256) as a template. Promoter and vector DNA fragments were mixed and directly used for transformation of RAK3605 (8). The sequences and maps of the resulting reporter plasmids are shown in Fig. S1.

SDS-PAGE Culture supernatants were mixed with an equal volume of SDS sample buffer, and proteins were denatured at 95°C for 10 min. A same amount (2−30 μL) of samples were loaded and run on a pre-cast SDS-PAGE gel (SuperSep Ace 5%−20% or 7.5%, Wako, Osaka, Japan), and proteins were stained with GelCode Blue Stain Reagent (Thermo Fisher Scientific, Waltham, MA). To determine N-terminal sequence, proteins were transferred onto a PVDF membrane, and bands were detected by staining with Coomassie Brilliant Blue-R25. The N-terminal sequence was analyzed by a contract analysis service. To remove *N*-glycans, proteins in culture supernatants were denatured in sample buffer at 95°C and treated with Endo H_f (New England BioLabs, Ipswich, MA) at 37°C for 2 h. Before SDS-PAGE, samples were incubated again at 95°C for 10 min.

Measurement of inulinase activity Inulinase activity was measured as sucrase activity. Culture supernatants were diluted 10-fold and reacted with 50 mM sucrose in McIlvain buffer (pH 5.0) at 45°C. To stop reactions, solutions were incubated at 100°C for 10 min. Glucose concentrations were quantified using a biosensor (BF-5/BF-30AS, Oji Scientific Instruments, Amagasaki, Japan) equipped with an auto sampler. Data are shown as mean ± SEM of three independent reactions. One unit of inulinase activity measured under the same conditions with 2% inulin as the substrate is equivalent to approximately six units of sucrase activity.

HPLC analysis of sugar concentration Sugar concentration in culture supernatants was measured using an HPLC system equipped with an RI detector (RID10A, Shimadzu, Kyoto, Japan) and a column (Shim-Pack SPR-Pb(G) 50 L × 7.8 mm, Shimadzu). The column temperature was set at 80°C, and water was used as the mobile phase. The error level in the measurements of 2% xylose was within 5%.

Determination of protein concentrations Protein concentration in culture supernatant was measured using the Quant-iT protein assay kit (Invitrogen, Carlsbad, CA, USA) with a fluorometer. To minimize autofluorescence of the YPX medium, culture supernatants were concentrated with Microcon (molecular cut-off 10 kDa, Merck Millipore, Darmstadt, Germany), washed three times with water, and recovered in water equivalent to the starting volume. YPX was also treated in the same way to subtract as a blank.

Luciferase assay Six transformants carrying the *INU1*p-yNLuc plasmid or *ACT1*p-yNLuc plasmid were individually cultured in YPD and YPX for 24 h. Cultures were diluted 100-fold for OD_{600} measurement and Luciferase assays. Luciferase activity was quantified using the Nano-Glo Luciferase assay system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Luminescence was measured using a GloMax-20/20 luminometer (Promega). Means and standard deviations of luciferase activity [relative luminescence unit (RLU)/OD_{600}] were calculated.

RESULTS

Enhanced production of extracellular proteins by *K. marxianus* DMKU 3−1042 in xylose medium To analyze the production of extracellular proteins by *K. marxianus*, the DMKU 3−1042 strain was cultured in media containing glucose (2%, 5%, or 10%) or 2% galactose, sucrose, xylose, xylitol, arabinose, lactose, or cellobiose. Culture supernatants were analyzed by SDS-PAGE (Fig. 1A). In 1-day culture, a band of approximately 90 kDa was detected in xylose medium. On the 2nd and 3rd days, bands of the same size were also observed in some of the other sugars, but the band intensity in the xylose medium was higher. However, the band was not detected in the medium containing 10% glucose. When the culture was extended to 5 days, production of the protein increased in xylose medium, but in the glucose medium many bands smaller than 90 kDa were observed (Fig. 1B). Production of specific extracellular proteins by *K. marxianus* in xylose medium has not been reported previously.

The abundantly produced extracellular protein is an inulinase To identify the protein produced in xylose medium, the N-terminal sequence of the protein was analyzed and was determined to be SGDSKAITXT (X means unknown). The sequence was homologous to a segment of the deduced amino acid sequence of the *INU1* gene in DMKU 3−1042. However, the expected molecular weight of the protein encoded by *INU1* is 62 kDa. The Inu1 protein sequence has 14 conserved *N*-glycosylation sites N-X-S/T (Fig. S2), suggesting that Inu1 is modified with *N*-glycans which would result in a higher molecular weight. To analyze *N*-glycosylation of the 90-kDa band, culture supernatant was treated with Endo H_f enzyme and analyzed by SDS-PAGE (Fig. 2A). Two bands were detected; one is Endo H_f and the other was approximately 60 kDa, indicating that the 90 kDa band was a highly *N*-glycosylated protein and the molecular weight of a deglycosylated protein would be similar to the deduced molecular weight of Inu1. To confirm whether the band is an inulinase, the *INU1* gene was disrupted, and extracellular proteins of the disruptant were analyzed. As shown in Fig. 2B, the 90-kDa band was not detected in the culture supernatant of the *Δinu1* strain, indicating that the protein that is abundantly produced in

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