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Increased Production of Exoinulinase in *Saccharomyces cerevisiae* by Expressing the *Kluyveromyces marxianus INU1* Gene Under the Control of the *INU1* Promoter

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Abstract *Aspergillus niger inuE* gene and *Kluyveromyces marxianus INU1* gene coding for exoinulinase were expressed in *Saccharomyces cerevisiae* under the control of *K. marxianus INU1* promoter. Recombinant *S. cerevisiae* expressing *K. marxianus* exoinulinase produced maximum 85 U/ml into culture medium, which was 9- to 14-fold higher than the activity produced by any other strain reported so far. In addition, *K. marxianus INU1* promoter produced 20-fold higher activity than *S. cerevisiae* glyceraldehydes phosphate dehydrogenase (GPD) promoter in *S. cerevisiae*.

Key words: *Aspergillus niger*, exoinulinase, *INU1*, *inuA*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*

Inulin is present in many edible plants including chicory, asparagus, and Jerusalem artichoke. The amount of inulin in such plants ranges up to 30% of plant mass, providing a high potential for microorganisms to use it as a carbon and energy source and for humans to use it as a useful source of fructose or oligofructose. Microorganisms need to produce exoinulinase, which hydrolyze inulin to fructose, and use this inulin as a carbon and energy source. Only a few efforts have been addressed to use inulin for production of oligofructose [4, 12]. Furthermore, chicory inulin has been directly used to produce oligofructose by a recombinant *Saccharomyces cerevisiae* strain expressing the *Aspergillus ficuum* endoinulinase gene (unpublished result).

Another utilization of inulin in edible plants is the production of alcoholic beverages by alcohol-fermenting microorganisms. Among many alcohol-fermenting microorganisms, *S. cerevisiae* is a representative in the alcohol fermentation industry. An effort to use plant inulin for production of ethanol has been addressed by the combined use of inulinase-producing microorganism and an alcohol-fermenting microorganism [10]. After saccharification of inulin by an inulinase-hyperproducing *Aspergillus niger* strain, alcohol fermentation was performed by using an *S. cerevisiae* strain [10]. Several microorganisms have been known to produce exoinulinase, including *Kluyveromyces marxianus*, *A. niger*, and *Penicillium purpurogenum* [for review, see ref. 11]; however, *S. cerevisiae* does not. In this study, the *inuE* gene and the *INU1* gene encoding exoinulinase of *A. niger* and *K. marxianus*, respectively, were expressed in *S. cerevisiae* to provide an *S. cerevisiae* with an ability to saccharify inulin.

All strains used in this work are listed in Table 1 and cultured in an appropriate medium that is described elsewhere. YPD and YPF were described previously [4, 12]. YPS and YPI contained the identical concentration of sucrose and inulin instead of glucose in YPD, respectively. Shuttle vectors, pRS414 having the *TRP1* gene and pYESTrp2, were purchased from Stratagene (La Jolla, CA, U.S.A.) and from KDR Biotech Co., Ltd. (Seoul, Korea), respectively. An *S. cerevisiae* strain was transformed by using polyethylene glycol and lithium acetate according to the procedure described elsewhere [3].

An *A. niger* genomic library was screened, which was constructed by insertion of the *Pst*I-digested *A. niger* genomic DNA fragments into the *Pst*I-site of pBluescript KS(+), by colony hybridization, using a 670 bp PCR product, and by isolating a 6.3 kb *Pst*I-digested fragment. A primer pair was designed on the basis of the nucleotide sequence of the *Aspergillus awamori* exoinulinase gene, of which the nucleotide sequences were 5'-AAC TAT GAC CAG CCT TA-3' (Aaw-inuAUP1) and 5'-CCA GTC TTT GAG GTT GT-3' (Aaw-inuALOW2). PCR (Ani-genomic PCR) was carried out by using *A. niger* genomic DNA as a

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Table 1. Strains used in this study.

Strain	Genotype or catalog no.	Source
Saccharomyces cerevisiae YSH 2.64-2C	*MATa trp1-92 his4 ura3-52 Δsuc2::URA3 mal10*	Ref. 1
Aspergillus awamori	KCTC 6970	KCTC[a]
Aspergillus niger	ATCC 6275	ATCC[b]
Kluyveromyces marxianus	ATCC 12424	ATCC

[a]Korean Collection for Type Cultures (Daejeon, Korea).
[b]American Type Culture Collection (Manassas, VA, U.S.A.).

template, and consisted of incubation at 95°C for 3 min, 35 cycles of incubation at 94°C for 30 sec, at 58°C for 30 sec and at 72°C for 2 min, followed by a final extension at 72°C for 5 min. Separately, a 1,655 bp cDNA generated by RT-PCR was digested with *Bam*HI and subcloned into pBluescript KS(+) which was digested with *Sma*I and *Bam*HI, producing pBCIA1. An *inuE* cDNA was also obtained by RT-PCR by using a primer pair, of which the sequences were 5'-ATC TGC AGT GGC TCG TCT TTT GA-3' (inuA RT11) and 5-GAG GAT CCA TGC TAC CAC ATG TA-3' (inuA RT12), and a single strand cDNA as a template. The single strand cDNA was synthesized by Expand™ Reverse Transcriptase (Roche Diagnostics GmbH, Mannheim, Germany) with a primer, 5'-TAG TTT ATT CAA TGA CCA-3' (inuA RT10). The PCR condition was identical to that in the above Ani-genomic PCR. The determination of nucleotide sequence of the *inuE* cDNA confirmed that the *inuE* cDNA did not have any error possibly appearing during PCR-amplification. The genomic DNA sequence in the present InuE ORF showed 98% identity to that of the previously reported *inuE* gene with 19 nucleotide differences and one amino acid difference [7].

An expression vector of the *K. marxianus INU1* gene in *S. cerevisiae*, pRCYIN1, was constructed. A 2,054 bp *Ssp*I-digested fragment of pRS414, having a centromere, was replaced with a 2,279 bp *Ssp*I-digested fragment of pYESTrp2, having 2 μ ori and the *TRP1* gene, thus generating pRSY. The 263 bp *CYC1* terminator was amplified by PCR, digested with *Sal*I and *Xho*I, and inserted into the pRSY pre-

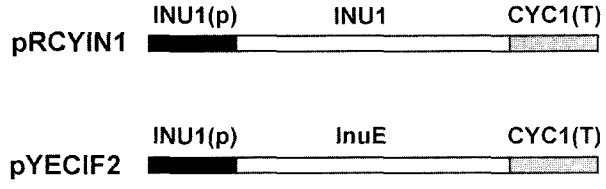

Fig. 1. Schematic presentation of expression cassettes of the *INU1* and *inuE* genes.
The *INU1* gene and the *inuE* cDNA were placed under the control of identical *K. marxianus INU1* promoter and *S. cerevisiae CYC1* transcription terminator. Close box (INU1(p)), the *K. marxianus INU1* promoter; open box (INU1), the *K. marxianus INU1* gene; open box (InuE), the *A. niger inuE* cDNA; shadow box (CYC1(T)), the *S. cerevisiae CYC1* transcription terminator.

linearized with the identical enzymes, yielding pRCY. The nucleotide sequences of primers used for amplification of the *CYC1* terminator were 5'-TCT GTC GAC CCG CAT CAT GTA ATT AG-3' (CYC1 up) and 5'-ATT CTC GAG GGG CCG CAA ATT AAA GC-3' (CYC1 lower), which were designed on the basis of the *CYC1* sequence in pYESTrp2. The PCR consisted of incubation at 94°C for 3 min, 35 cycles of incubation at 94°C for 30 sec, at 57°C for 30 sec and at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The *INU1* gene [7] coding for exoinulinase was amplified by PCR using *K. marxianus* genomic DNA as a template, digested with *Sac*I and *Sal*I and inserted into a pRCY digested with identical enzymes, thus generating pRCYIN1. The nucleotide sequences of primers were 5'-TTG GAG CTC TTA GTG CGT GAA AGT ATC-3' (INU1 upper) and 5'-TTG GTC GAC GTA AAG TAA GCA GAT CAG-3' (INU1 lower). The PCR was carried out at 94°C for 3 min, 35 cycles of incubation at 94°C for 30 sec, at 57°C for 30 sec and at 72°C for 2 min 30 sec, and a final extension at 72°C for 5 min.

An expression vector of the *A. niger inuE* gene, pYECIF2, was also constructed. The PCR-amplified *K. marxianus INU1* promoter was inserted into the pBCIA1 digested with *Cla*I and *Pst*I, generating pBCIA2. The nucleotide sequences of the primer pair used for amplification of the *INU1* promoter were 5-GTT CCG CGG GTC ACG ACG TTG TAA-3' (inuP upper) and 5-TTC TGC AGT CTC TTG TAA TTG ATA ACT GAA-3' (inuP low2). The PCR was performed under the conditions identical to that for RT-PCR to isolate the *A. niger inuE* cDNA. A 2,384 bp fragment obtained by digestion of the pBCIA2 with *Bam*HI and *Cla*I replaced the *INU1* promoter and the *inuB* gene in pYESINU2, thus generating pYECIF2.

The pRCYIN1 contains a fragment, having the *INU1* promoter, the Inu1 ORF, the *CYC1* terminator, and the 2μ ori. The pYECIF2 contains the features identical to pRCYIN1, except the InuE ORF without an intron in place of the Inu1 ORF. Each of these two recombinant plasmids was introduced into an *S. cerevisiae* strain that produced the highest endoinulinase activity among four strains analyzed previously [12].

Previous study indicated that the production of the endoinulinase is influenced by the carbon/energy source supplied in the medium, when the *A. ficuum* endoinulinase

gene is expressed under the control of the *K. marxianus INU1* promoter [12]. Therefore, in the present study, the exoinulinase activities of the recombinant *S. cerevisiae* strains and *K. marxianus* were compared on medium containing different carbon sources: The exoinulinase activity in cell-free culture medium was analyzed by measuring the concentration of reducing sugars produced by the method described previously [6]. Thus, *S. cerevisiae* strains were grown for 72 h at 30°C in YPD. The standard reaction mixture contained 10 µl cell-free culture medium, 1% (final concentration, w/v) inulin in 100 µl H_2O, and was incubated at 52°C for 1 h. The amount of liberated reducing sugars was measured using authentic fructose as a standard. When necessary, liberation of fructose from inulin by exoinulinase was confirmed by high performance liquid chromatography (HPLC) [4, 12]. One unit of the exoinulinase activity was defined as the amount of enzyme to produce 1 µmole of reducing sugars in one minute under the standard reaction conditions. This unit definition was identical to those used in previous works [2, 7]. As shown in Table 2, the recombinant *S. cerevisiae* strain, having the *INU1* gene, in any medium produced higher amount of exoinulinase than the *K. marxianus* strain, from which the *INU1* gene and its cDNA were isolated, producing maximum 9-fold higher exoinulinase activity than the *K. marxianus* strain. Such high exoinulinase productivity in the recombinant *S. cerevisiae* than *K. marxianus* was probably due to the difference in the copy number of the exoinulinase gene in both strains: *K. marxianus* contains one copy, while the recombinant *S. cerevisiae* contains the gene in multi-copy. Such possibility was supported by previous results that a recombinant *S. cerevisiae* strain expressing the *A. ficuum* endoinulinase gene produced 20-fold higher endoinulinase, when the gene was in a multi-copy plasmid rather than in a single-copy plasmid [4, 12].

The recombinant *S. cerevisiae* strain having the *INU1* gene produced higher exoinulinase activity than the *S. cerevisiae* strain having the *inuE* gene in any medium examined, although both genes were under the control of the *K. marxianus INU1* promoter (Table 2). At present, it is still uncertain why the *INU1* gene produced higher activity than the *inuE* gene in the recombinant *S. cerevisiae* strain.

Furthermore, the recombinant *S. cerevisiae* strain, having the *INU1* gene, produced the exoinulinase activity of 85 U/ml on YPD, while the strain produced less amount of the activity on YPF, YPS, and YPI (Table 2). The *INU1* promoter used in this study is known to have the putative binding site of the *S. cerevisiae* MIG1 repressor of transcription in the presence of glucose [1, 8, 9]. Therefore, the exoinulinase activity produced from the recombinant *S. cerevisiae* strain on YPD was expected to be lower than on other media. However, as seen in Table 2, the productivity was higher on YPD than any other medium examined. This unexpected observation might have been partly due to exhaustion of the glucose added to the medium (data not shown). Consumption of glucose and liberation of fructose from inulin was confirmed by HPLC [12]. Moreover, the activity of 85 U/ml was 5-fold higher than that produced in *Pichia pastoris* expressing the *A. niger inuE* gene [7]. The recombinant *P. pastoris* produced 16 U/ml into cell-free culture medium. When the exoinulinase activity of the recombinant strain was measured at 40°C for 30 min, as was in the previous work [7], but not at 52°C for 1 h, the activity was 71.9 U/ml, which was also much higher than that obtained in the recombinant *P. pastoris*. This activity difference might also be due to the copy number of the exoinulinase gene in the recombinant *S. cerevisiae* strain and the recombinant *P. pastoris*: In the recombinant *P. pastoris*, the copy number of the gene exoinulinase was one, while it was multi-copy in the recombinant *S. cerevisiae* strain. Furthermore, the activity of 85 U/ml was 20-fold higher than that in the previous work where *S. cerevisiae* expressed the *K. marxianus INU1* gene under the control of glyceraldehydes-3-phosphate dehydrogenase (*GPD*) promoter [2], which is known as

Table 2. The exoinulinase activities of the strains on various media.

Strain and plasmid	Enzyme activity (U/ml)[a] in liquid medium			
	YPD	YPF	YPS	YPI
K. marxianus	9.3±2.8	6.3±2.2	5.2±2.5	8.2±2.5
(absorbance[b])	(0.56±0.02)	(0.87±0.01)	(0.65±0.01)	(0.96±0.02)
S. cerevisiae[c]				
pRCYIN1	85.4±4.1	68.2±3.3	26.8±3.5	19.1±2.9
(absorbance)	(0.78±0.07)	(0.70±0.02)	(0.07±0.04)	(0.68±0.04)
pYECINF2	5.9±2.8	4.2±2.3	5.1±2.70	4.4±2.8
(absorbance)	(0.82±0.00)	(0.79±0.02)	(0.66±0.02)	(0.40±0.010)

The enzyme activity was measured in three experiments and expressed as average±standard deviation.

[a]One unit of the exoinulinase activity was defined as the amount of enzyme to produce 1 µmole of reducing sugars in one minute under the standard reaction conditions.

[b]Absorbance was measured at 600 nm.

[c]*S. cerevisiae* YSH 2.64-2C strain having the recombinant plasmid.

one of the strongest promoters in *S. cerevisiae*. Also, the *K. marxianus* Inu1 signal sequence worked well enough to secrete the exoinulinase synthesized. In conclusion, our results suggest that the *K. marxianus INU1* promoter and the Inu1 signal sequence can be used to produce secreted proteins in *S. cerevisiae,* and that the *K. marxianus INU1* gene produces higher activity than the *A. niger inuE* gene, when expressed in *S. cerevisiae.*

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