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Yeast Cloning Vectors and their Application to the Development of Starch-fermenting Yeast

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효모 Cloning Vector와 전분발효 효모의 개발

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Abstract

Transformed, hybrid strains of the yeast *Saccharomyces* capable of simultaneous secretion of both glucoamylase and α-amylase have been produced. These strains can carry out direct, one-step assimilation of starch with conversion efficiency greater than 93% during a 5 day growth period. One of the transformants converts 92.8% of available starch into reducing sugars in only 2 days. Glucoamylase secretion by these strains results from expression of one or more chromosomal STA genes derived from *Saccharomyces diastaticus*. The strains were transformed by a plasmid(pMS12) containing mouse salivary α-amylase cDNA in an expression vector containing yeast alcohol dehydrogenase promoter and a segment of yeast 2μ plasmid. The major starch hydrolysis product produced by crude amylases found in culture broths is glucose, indicating that α-amylase and glucoamylase act cooperatively. (IN ENG)

Introduction

Yeasts have been used by human being for several thousand years as brewing and bread baking agents. In the present day, yeast is the one of the most important and useful microorganism in industry to produce foods, medicines, and fuel alcohol. Also yeast is the fastes tgrowing research subject in biology to study some extremely complex phenomena specific to eukaryotes. This article describes several yeast cloning vectors and reports their application to the development of starch-fermenting yeast.

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Yeast Cloning Vectors

There are five different kinds of yeast plasmid vector systems depending on the modes by which DNA can be maintained in yeast. The plasmids that can only be maintained by integration into homologous chromosomal DNA are designated YIp(yeast integrating plasmid); those that use a fragment of the 2μ circle plasmid for maintenance are called YEp(yeast episomal plasmid); the plasmids that are maintained because they contain an autonomously replicating sequence are called YRp(yeast replicating plasmid); the plasmids that contain a functional centromere are called YCp(yeast centromere plasmid); and the linear plasmids containing

telomeres are called YLp(yeast linear plasmid). All systems employ selectable markers(most commonly LEU2, HIS3, URA3, and TRP1) that can be selected in yeast mutant strains for that gene. Except YLp plasmid vector, all the vectors are circular from containing both bacterial sequences that signal DNA replication in *E. coli* and sequences that signal DNA replication in yeast. These vectors are called shuttle vectors.

YIp Plasmid Vectors

In yeast, plasmid integration occurs by homologous recombination. For example, YIp5 contains URA3 and can be integrated at the chromosomal site of URA3.[1] Although the frequency of transformation is extremely low(1~10 transformants/μg DNA/10^7 cells),[2,3] the transformants are very stable when compared to transformants of YRp or YEp plasmids. The rate of segregation of YIp plasmids is usually much less than 1% per generation of growth in a non selective medium.

YEp Plasmid Vectors

Most strains of yeast naturally contain an autonomously replicating plasmid called the 2μ circle. The YEp plasmid vector contains certain portions of the 2μ circle, transform yeast at high frequency(10^3—10^4 transformant/μg DNA/10^7 cells), and replicate autonomously in yeast cells.[4] This plasmid is more stable than YRp plasmid vector; between 60% and 95% of the cell contain the plasmid after 10 generations of non selective growth.[5] Cells that contain the plasmid have relatively high copy number(20~50 copies/cell). These vectors generally used for constructing libraries to clone genes by complementation. The presence of native 2μ circle DNA in the host strain enhances the stability of these vectors.

YRp Plasmid Vector

High frequency transformation(10^3—10^4 transformant/μg DNA/10^6 cells) can be obtained when certain chromosomal sequences are included in the vector. These sequences allow autonomous replication of the vector and are named ARS for autonomously replicating sequence, which are involved in the initiation of DNA replication.[6] Plasmids that contain chromosomal ARS sequences are generally unstable.[1] After growth in non selective conditions(10 generations), 5% of the cells contain the plasmid. Occasional stable variants can be found among transformants; these appear to be cases in which the plasmid, including the ARS segment, has integrated into a homologous region in the same way that YIp plasmids do.[6,7] Cells that contain the plasmid have relatively high copy-number (20~50 copies/cell). Yeast libraries can be constructed with YRp plasmid for cloning genes by complementation.

YCp Plasmid Vectors

When yeast centromere(CEN) sequences are added to the plasmids containing chromosomal ARS, stability increases such that 90% of the cells contain the plasmid after 10 generations.[8] In the eukaryotic cell, the CEN sequences ensure the attachment of the chromosomes to the spindle fibers of the mitotic apparatus, and therefore effect the equal segregation of the chromosomes when the cell divides. The YCp vector is useful for constructing libraries and cloning genes that may be lethal when present in more than one copy per cell.

YLd Plasmid Vectors

Linear plasmids have been constructed by cloning telomere sequences from cilate or yeast into plasmids to create autonomously replicating linear molecules.[9] Telomere is the name for the end of a chromosome and the two telomere sequences per chromosome are believed to give a linear DNA the ability to replicate.[10] These linear plasmids are less stable than circular centromere-bearing plasmids: approximately 40% of the cells contain 20~40 copies after 10 generations. The linear molecules can be further stabilized by adding a yeast centromere sequence

and also increasing the total size of the plasmid.[11] The resultant molecule, a minichromosome present in one copy, is the most stable vector so far described, but it is still less stable than a native chromosome. This minichromosome is useful for studying sequences necessary for proper chromosome function.

Expression and/or secretion vectors

Based on above mentioned cloning vectors, derivative vectors could be constructed that aid in the expression and/or secretion of proteins in yeast. Some expression vectors containing strong constitutive promoters such as the promoter of yeast alcohol dehydrogenase[12] are useful for over-expression of a gene product in yeast cells. Other expression vectors utilize regulated promoters from genes such as acid phosphatase.[13] A circular plasmid that contains the gene encoding the signal peptide of the mating pheromone α-factor was used to secrete hybrid proteins.[14]

Development of Starch-fermenting Yeast

The conversion of starch biomass to industrial and fuel ethanol by *Saccharomyces cerevisiae* or *S. carlsbergensis* employs a three-step process: (i) liquefaction of starch with bacterial α-amylase, (ii) enzymatic saccharification of the liquefied starch to produce fermentable sugars, and (iii) fermentation of the sugars. The commercial enzymes used for starch degradation represent a significant cost in the production of fermentation alcohol. Several yeast of genera other than *Saccharomyces* assimilate starch, and a number of these organisms secrete enzymes with debranching and/or α-amylase activity.[15] Recent interest in such yeasts has focused on their potential for one-step starch fermentation.[16, 17] However, these amylolytic yeasts are generally not suitable of alcohol production because they have a low tolerance for ethanol and exhibit slow fermentation rates.[17]

On the other hand, one amylolytic yeast, *Saccharomyces diastaticus*, does exhibit high tolerance for alcohol and high fermentation rates.[17,18] Laluce and Mattoon[18] examined a strain that produced 12%(vol/vol) ethanol in a four-day fermentation. *S. diastaticus* is very closely related to *S. cerevisiae* genetically, and the two species are readily hybridizd.[18,19] Their primary differenence is that *S. diastaticus* secretes a glucoamylase whereas *S. cerevisiae* lacks this ability. Glucoamylase secretion is determined by the presence of one or more unlinked glucoamylase structural genes in *S. diastaticus*.[19~22] Three such genes, STA1, STA2, and STA3, have been identified[19,20] and cloned.[21,23~26] The nucleotide sequence of the STA1 genee has been determined.[27]

Laluce and Mattoon[18] evaluated a variety of *S. diastaticus* strains for direct conversion of starch and dextrins to ethanol. Through a combination of strain selection, hybridization, and systematic optimization of fermentation conditions, up to 80% conversion of Lintner starch was attained using *S. diastaticus*. The residual 20% carbohydrate represents some type of limit dextrin which is refractory to hydrolysis by *S. diastaticus* glucoamylases. However, this refractory starch residue can be almost entirely eliminafed by prior treatment of starch with α-amylase. When starch was first digested by commercial α-amylase(Taka-therm, Miles Laboratories), 97% conversion of starch was achieved with *S. diastaticus*.[18]

Several laboratories have introduced heterologous α-amylase genes derived from various organisms into *Saccharomyces cerevisiae* to produce transformants which secrete active α-amylase into the culture medium.[28~32] For example, Thomsen[30,31] constructed chimeric plasmids containing mouse salivary α-amylase cDNA under control of the promoter of *S. cerevisiae* alcohol dehydrogenase I(ADHI) gene ADCI. A transformant of *S. cerevisiae* bearing plasmid pMS12 was capable of direct fermentation of starch from various sources, but conversion of carbohydrate to alcohol was not efficient, varying

from 10 to 50%,[31]

The present report describes the preparation of a yeast strain which secretes both α-amylase and glucoamlase. This strain was obtained by transforming a *S. diastaticus* derivative with the mouse salivary α-amylase plasmid pMS12. With this yeast 97% degradation and 93% utilization of Lintner starch has been obtained.

Yeast strains

Strains containing both STA (glucoamylase) genes and the transformant selection marker TRP1 were constructed by crossing *S. cerevisiae* strain SHU 32a with *S. diastaticus* strain 5301-17B and *S. cerevisiae* strain SHU32α with hybrid strain CL1-17B. The resulting diploids were sporulated and segregants, KK1-R1 and KK2-R1 exhibiting both tryptophan auxotrophy and glucoamylase secretion were selected.

Plasmid

Plasmid pMS12,[31] containing a cDNA coding for mouse salivary α-amylase in yeast expression vector pMA56[33] was generously provided by Dr. Karl K. Thomsen, Carlsberg Laboratory, Valby, Denmark. The expression vector contains the *E. coli* origin of replication and the β-lactamase gene of pBR322, a segment of yeast 2μ DNA containing an origin of replication, the yeast TRP1 gene and the promoter of the alcohol dehydrogenase I gene, ADCI. In chimeric plasmid pMS12, the α-amylase cDNA has been inserted by means of an EcoR1 linker downstream of position 14 of the ADCI gene. The cDNA includes the mouse α-amylase signal peptide which is 15 amino acid residues long. Plasmid pMS12 was maintained in *E. coli* C600 SF8 grown in LB medium containing an ampicillin concentration of 50 μg/ml. Amplification and extraction of plasmid was performed as described by Maniatis et al.[34]

Media and Growth

Complete buffered starch medium (BYPS4) contained 1% Difco yeast extract(Y), 2% Difco peptone (p), and 4% Lintner starch, and Succinic acid buffer (0.1M, pH 4.2). Minimal medium contained 0.6% Difco yeast nitrogen base(without amino acids), 2% dextrose (D) and nutritional supplements, as required.[35] All growth experiments were conducted at 30°C. Stock cultures were grown on YPD except for transformants carrying pMS12 which were grown on minimal medium lacking tryptophan. For the measurement of residual carbohydrate, 2 loopfuls of cells previously grown on minimal medium for 2 days were used to inoculate a 500ml flask containing 150ml complete buffered starch medium and incubated on a 30°C rotary shaker for 5 days at 300 RPM.

Transformation

Yeast cells were transformed according to the method of Ito et al.[36] using lithium acetate. Since the yeast strains SHU32a, KK1-R1, and KK2-R1 each carries a mutation in the TRP1 gene, and can be complemented by the TRP1 gene carried on the plasmid pMS12, transformants were selected on minimal medium lacking tryptophan.

Carbohydrate assay

Glucose was assayed with a glucose oxidase/peroxidase method using PGO-enzymes supplied by Sigma (St. Louis, MO). Reducing sugar was determined by the colorimetric method using 3, 5-dinitrosalicylic acid,[37] employing glucose to make a standard curve. Residual starch was hydrolysed with 1 N HCl for 35 min at 97°C. From the amount of resulting reducing sugar, the residual starch content was calculated.[38] The percentage of sugar taken up by yeast during growth was determined using the following equation: % sugar uptake=100−% residual starch−% residual sugar

Amylase assay

The reaction mixture for the enzyme assay contained 0.2ml 1.6% Lintner potato starch, 0.1 ml sodium acetate buffer(1M, pH 5), and 0.7 ml centrifuged culture fluid as crude enzyme

solution. After a 30min incubation at 55°C, the reaction was stopped by immersing the tube containing the reaction mixture in a boiling water bath for 10min. After cooling the tube, glucose content was determined.

Sugar uptake and residual carbohydrate resulting from growth of transformed and untransformed strains

Because secreted amylases are present continuously during growth of yeast cells, the carbohydrate content of the culture broth at a given time will reflect both the type and activity of amylolytic enzymes present as well as the ability of the cells to assimilate various starch hydrolysis products. Table 1 presents an analysis of residual carbohydrates present in 5-day culture media after growth of the various transformants and their untransformed counterparts. Sugar uptake repeesents the difference between the total carbohydrate still in the culture broth after the 5-day growth period and the initial starch added to the culture broth.

As expected, untransformed *S. cervisiae* strain SHU32a consumed little or no sugar, and almost all the starch remained at the end of the incubation period. In contrast, less than 30% of the starch remained in the broths obtained from cultures of the two untransformed hybrid KK1-R1 and KK2-R1, reflecting the activity of the glucoamylase secreted by these strains. The very low residual sugar content of the broths indicates that the primary starch hydrclysis product is glucose, which is rapidly assimilated by the yeast cells. In a separate experiment for the assay of amylases secreted by KK1-R1 and KK2-R1 yeast strains, the main product of the enzyme reaction was found to be glucose. Comparison of the results obtained with the three types of transformants reflects the differences in the type or types of amylases secreted. The transformant of SHU32a, which secretes only α-amylase utilized less sugar(77.0%) than the hybrid transformants(94%). Moreover, the SHU32a transformant left a substantial residue of residual starch(12.9%) and unutilized reducing sugar(10.2%) in the culture medium. In contrast, hybrid transformants left only about 3% unused starch and 3% unused reducing sugar. Clearly, then, hybrid transformants secreting both types of amylases can utilize starch much more completely than a strain secreting only α-amylase or glucoamylase.

The relatively high concentration(10.2%) of residual sugar in culture medium of the SHU32a transformant secreting only α-amylase is consistent with the observations of Panchal[39] that this enzyme produces significant quantities of maltotetraose and larger oligosaccharides which cannot be transported into the yeast cell.

In a previous report, Matton et al.[21] reported that direct conversion of 4% Lintner starch to ethanol was no greater than 80% even after 12 days fermentation by *S. diastaticus*. However, pretreatment of starch with α-amylase permitted more than 95% conversion by the same organ-

Table 1. Sugar uptake and residual carbohydrates in culture media after growth for five days

Yeast Strain	Carbohydrate(%±SD)		
	Sugar taken up	Residual sugar*	Residual starch
SHU32a-untransformed	1.4±1.70	1.1±0.07	97.5±1.77
transformed	77.0±0.26	10.2±0.26	12.9±0.53
KK1-R1-untransformed	69.4±2.74	1.6±0.08	29.0±2.83
transformed	93.8±0.41	3.2±0.30	3.0±0.71
KK2-R1-untransformed	69.8±0.92	2.3±0.74	27.9±0.18
transformed	93.6±0.09	3.8±0.26	2.6±0.18

* Residual sugar measured as reucing sugadr.

ism. The present study demonstrates that by constructing a yeast transformant capable of secreting both α-amylase and glucoamylase, direct conversion of more than 93% of starch in 5 days can be attained. Thus, it is now possible to attain almost the same efficiency of conversion in one step as was previously attained in two steps.[18,21] Moreover, by using such a yeast strain, the cost of commercial α-amylase in conversion of starch to ethanol could be eliminated.

Mitotic stability of yeast strains transformed with plasmid pMS12

It is generally observed that yeast transformants bearing plasmids carrying the 2μ origin of replication exhibit varying degrees of mitotic instability. That is, during many generations of growth under non-selective conditions cells with plasmids are gradually diluted out of the population. The stability of the various transformants obtained with plasmid pMS12 was examined, and the results are presented in Table 2. Cells were grown on BYPS4 medium which contains tryptophan as a component of yeast extract and of peptone. Therefore, there was no selective pressure to retain the TRP1 gene residing on the plasmid. However, since starch was the primary carbon source, there is some selectve pressure to retain the ability to produce amylase. As shown in Table 2, plasmid loss, as measured by retention of tryptophan prototrophy, occurs progressively during growth of the three different transformants. The more limited loss of plasmid by the *S. cerevisiae* (SHU32a) transformants may reflect the fact that the only source of amylolytic activity in this strain is the plasmid α-amylase gene, whereas cells of KK1-R1 and KK1-R2 can produce glucoamylase even after they have lost all plasmid. An alternative explanation for the difference between the plasmid stability of the hybrid trnnsformants and the SHU32a transformant could be that fewer generations occurred in the culture of the latter strain, which does grow more slowly than the hybrid transformants.

Table 2. Mitotic stability of α-amylase plasmid pMS12 in different yeast transformants*

Yeast Transformants	Mitotic stability** (%)after	
	3 days	5 days
SHU32a/pMS12	86.2±2.19	72.5±0.35
KK1-R1/pMS12	54.0±9.05	45.9±2.97
KK2-R1/pMS12	51.8±8.13	49.7±0.50

* The mitotic stability was determined after growing the yeast cells aerobibally in 150ml BYPS4 for 3 or 5 days

** Mitotic stability(%)=100×(No. of colonies on tryptophanless minimal medium/No. of colonies on minimal medium with tryptophan)

Further improvement in strains possessing both glucoamylase genes and α-amylase genes could be made by incorporating several repeated genes of the α-amylase into a centromere vector (YCp),[40] a minichromosome(YLp) or by integrating the gene(YIp), together with the attached ADCI promoter, into a chromosome of the host cell to obtain stable strain. Althouh Filho et al.[28] reported a plasmid vector containing 2μ DNA, a LEU2 gene and mouse pancreatic α-amylase cDNA was integrated into a yeast chromosome with low frequency, the presence of 2μ sequences may destabilize such integrated fragments.

In conclusion, efficient one-step starch utilization has been achieved by constructing *Saccharomyces* strains capable of secreting both glucoamylase and α-amylase simultaneously.

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