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REVIEWS

Invertase Overproduction May Provide for Inulin Fermentation by Selection Strains of *Saccharomyces cerevisiae*

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Abstract—In some recent publications, the ability of selection strains of *Saccharomyces cerevisiae* to ferment inulin was attributed to inulinase activity. The review summarizes the literature data indicating that overproduction of invertase, an enzyme common to *S. cerevisiae*, may be responsible for this phenomenon.

Keywords: yeasts, *Saccharomyces cerevisiae,* β-fructosidase, invertase, inulinase, *SUC* genes, catabolite repression, inulin, polyfructosides

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There are extraordinary reports from several laboratories on selection of *Saccharomyces cerevisiae* strains utilizing inulin [1–6] and, according to a number of authors, possessing inulinase, a specific enzyme hydrolyzing inulin. Although β-fructosidases (invertases) of *Saccharomyces* yeasts have been under study for over 150 years, detection of another β-fructosidase with inulinase activity has never been reported. In this review, we summarize the literature data to attribute the so-called inulinase activity in *S. cerevisiae* to overexpression of the common invertase.

Inulin is a polysaccharide consisting of several fructose residues linked by a β-1,2 bond. It is widely used in medicine, the food industry, and biotechnology [7, 8]. Inulin-rich raw materials, such as girasol (*Helianthus tuberosus*), inula (*Inula helenium*), artichoke (*Cynara* species), dahlia (*Dahlia* species), chicory (*Cichorium* species), asparagus (*Asparagus racemosus*), yacon (*Smallanthus sonchifolius*), and blue agave (*Agave tequilana*), can replace starch, glucose, maltose, and sucrose in fermentation processes after hydrolysis by a specific β-fructosidase (inulinase). The reference yeast inulinase is produced by the representatives of the genus *Kluyveromyces*, in particular, *K. marxianus* (syn. *K. cicerisporus, K. fragilis*, and *K. marxianus* var. *bulgaricus*) [9, 10]. Since these yeasts are not traditional for food biotechnology, the well-proven *S. cerevisiae* could be more appropriate for food and medical applications. However, their invertase hydrolyses inulin at a low rate. Are there ways to overcome these difficulties? Genetics of *S. cerevisiae* β-fructosidase can answer this question.

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POLYMERIC β-FRUCTOSIDASE GENES *SUC* AND REGULATION OF THEIR EXPRESSION

It is well-known that *S. cerevisiae*, as well as its sibling species *S. arboricola, S. bayanus, S. cariocanus,* and *S. mikitae*, do not consume inulin; *S. kudriavzevii* is an exception [11]. As we have already noted, invertase hydrolyzes inulin at a low rate and therefore cannot provide for the growth of these yeasts on inulin.

Numerous studies conducted in many laboratories revealed β-fructosidase of *S. cerevisiae* to be encoded by polymeric cumulative very closely related *SUC* genes (*SUC1–SUC5* and *SUC7–SUC10*) located in telomeric mobile regions of a number of chromosomes [12–16]. Two mRNAs (1.9 and 1.8 kb-long) differing by their 5'-ends are transcribed from each *SUC* gene. Only the larger mRNA encodes the signal peptide that determines secretion of the glycosylated invertase dominating by its content and function, while the smaller mRNA encodes a minor intracellular invertase with unknown role [17].

All *S. cerevisiae* strains possess an active *SUC2* gene or, rarely, a silent sequence (pseudogene) $suc2^0$ [16, 18, 19]. Cultured *S. cerevisiae* strains, in contrast to environmental isolates, contain different sets of *SUC* genes [20–22]. No special *SUC* genes with inulinase activity have been discovered. Nevertheless, a priori one can state that a dramatic increase in invertase content may lead to rapid degradation of inulin and yeast growth on it. Clearly, *S. cerevisiae* growth on inulin should depend on the regulation of β-fructosidase synthesis. There are several ways to induce overexpression of the *SUC* genes. As early as at the dawn of enzymology, Willstatter [23] proposed a method to enrich brewing yeasts with invertase by slowly supplementing their

biomass with sucrose. Numerous subsequent studies demonstrated β-fructosidase synthesis in most *S. cerevisiae* strains to depend on catabolite repression by the products of sucrose hydrolysis, glucose and/or fructose. In one of the first works on the mutants not sensitive to catabolite repression, these two-step UV mutants were shown to be capable of growth on raffinose synthesizing 20–30 times more invertase than the initial yeast. Invertase made up 1–2% of the total cell protein [24]. In another example, in *drg2* mutants with low sensitivity to catabolite repression obtained using chemical mutagens (nitrosoguanidine and ethyl methanesulfonate), invertase overproduction was also noted; the initial strain was of the *DGR2 SUC3* genotype [25]. Six genes (*SNF1–SNF6*) responsible for glucose repression of the *SUC* loci have been identified [26, 27]. Suppressor *ssn6* mutation of a mutant *snf1* allele leads to constitutive (not sensitive to glucose) high level of invertase synthesis in strains of the *SUC2* genotype. Even higher level of synthesis was reached in yeasts of the *ssn6-1 SNF1 SUC2* genotype, which was almost twice that of the wild type yeast (*SSN6 SNF1 SUC2*) under conditions of glucose derepression and 300-fold under repression with glucose [28]. Existence of a regulatory region close to the sequence encoding β-fructosidase has been demonstrated by the example of the *SUC2* locus, for the first time [29]. The product of the *SSN6* gene is a repressor of the *SUC2* gene.

Deletions in the regulatory region lead to derepression of invertase synthesis. The low 10% level of invertase synthesis by the *SUC7* strain, if compared to the *SUC2* strain at the same genetic background, can be caused by differences in the regulatory region [30]. Further studies [31, 32] also demonstrated a connection between the regulatory elements and invertase production. Two different types of TATA boxes were identified: the more actively expressed genes (*SUC1, SUC2*, and *SUC4*) had TATAAA sequence, while the less actively expressed genes (*SUC3, SUC5*, and *SUC7*) had the TACAAA sequence. Transformation of the strains possessing different *SUC* genes with plasmids containing multiple copies of the regulatory regions of the *SUC4* gene can increase invertase activity under conditions of both repression and derepression [33].

S. CEREVISIAE MUTANT CAN UTILIZE INULIN

An important genetic work of Grossman [34] on isolation of *S. cerevisiae* mutants utilizing inulin is little known, since it had been published as an abstract in a difficult to access source and has become available online only recently (http://www.uwo.ca/biology/YeastNewsletter/Index.html).

Let us cite the relevant part of the report [34]: "A new gene *SUC7* has been identified. It is present in a silent form in all strains studied so far. Active mutant alleles can be induced by UV irradiation and then selected as raffinose fermenters in the case of *suc*^{0} strains (no invertase made) or as inulin utilizers in. e.g., *SUC4* strains. ... Inulin cannot be utilized sufficiently well to support growth of our raffinose fermenting strains. Therefore, it is possible to select mutants utilizing inulin efficiently. This way, it was hoped to isolate mutants with regulatory effects increasing the formation of invertase. In fact, a dominant mutant was found which formed on a raffinose medium 18–20 units of invertase, whereas the parent *SUC4* strain had only 4–6. The inulin-utilizing mutant was a double mutant with an additional active *SUC7*-allele and another mutation leading to the excretion of large amounts of invertase already during log-phase." Thus, Grossman clearly indicated that a mutant with overexpression of invertase may be obtained by selection according to growth on the medium containing inulin. It should be noted that this method had a prototype. Inulinase principle of selection for invertase overproducers is the same as on the medium with raffinose in the presence of 2-deoxy-D-glucose (2-DG) [25]. The trisaccharide raffinose is also a β-fructoside, which may be hydrolyzed by invertase to fructose and a disaccharide melibiose. However, raffinose has low affinity to invertase, compared to sucrose, and additional 2-DG inhibition of invertase synthesis blocks raffinose fermentation [25, 35]. Therefore, the *drg* mutants capable of growth on raffinose in the presence of 2-DG exhibited overproduction of invertase [25, 26].

Let us analyze the relevant publications on industrial selection strains capable of growth on inulin, taking into account genetic control overexpression of the *SUC* genes in *S. cerevisiae*. The patent [1] reports a summary on the development of an Inu$^+$ mutant (VGSh-2) using a combination of UV irradiation and chemical mutagen treatment in the XII race of *S. cerevisiae*. In the VGSh-2 mutants, a 2.5–3-fold increase in β-fructosidase activity and the ability to synthesize inulinase were noted. In a subsequent publication by the authors [2], synthesis of endoinulinase by the producer and isolation of the enzyme were reported. The presented data on enzyme isolation contained no information on the separation of inulinase and invertase activity. The XII race, which was obtained from the All-Russian Collection of Microorganisms (VKM) in Pushchino, Russia, presumably under the number VKM Y-1169 was used as a starting strain. Earlier [22], we used molecular karyotyping and subsequent Southern hybridization of the *SUC2* probe with chromosomal DNA to determine the genotype of the strain as *SUC1 SUC2 SUC7 SUC8*. Importantly, distiller's strains in general have many *SUC* genes [21, 22]. The genotype of the XII race makes it possible to expect its high invertase activity, which, obviously, can become still further in the regulatory gene mutants.

Strain G of baker's yeast *S. cerevisiae* ZS was selected on a selection medium [3]. Strain G is capable of growth on medium with inulin as a single carbon source. Moreover, unlike the previous strain VGSh-2

[1, 2], strain G could ferment inulin. Growing of strain G on various carbon sources (glucose, sucrose, and inulin) demonstrated the constitutive synthesis of β-fructosidase. According to the work [36], many *SUC* genes are present in *S. cerevisiae* baker's yeast; it is therefore not surprising that the authors [3] easily isolated the variant growing on inulin under selection conditions.

RARE ENVIRONMENTAL STRAINS FERMENTING INULIN

Recently, yeasts capable of active utilization of polyfructosides with different degrees of polymerization have been discovered among non-industrial *S. cerevisiae* strains [4–6]. The aim of the search for such yeasts was to find ethanol producers for fermentation of inulin-containing raw materials. Here it should be noted that girasol, as well as other plants, contains both low molecular weight crystal polyfructosides and high molecular weight polyfructosides, including inulin. In general, a series of polyfructoside homologues of increasing molecular weight occurs in plants. For example, β-levulin, secalin, and graminin contain two, four, and ten fructose residues, respectively. There are also irisin, asparagosin, etc. [37].

The KCCM 50549 strain, which, in contrast to strain NCYC 625 utilizing only polyfructosides containing up to six fructose residues (easily available for hydrolysis by common invertase), was capable of utilizing most of artichoke fructosides containing up to 15 fructose residues, was discovered in South Korea [4]. The KCCM 50549 inulin-fermenting strain, according to the authors, formed 1.6 times more ethanol from raw artichoke than the NCYC 625 strain. Ethanol yield in a 5-L fermenting vessel was 36.2 g/L in 36 h; polyfructosides conversion to ethanol was 70% of the theoretically expected value.

Screening of the collection of *S. cerevisiae* strains isolated in China from various environmental sources (totaling 65) resulted in discovery of four strains utilizing inulin: JZIC, BJL3, BJL7, and TABL2 [38]. Strain JZIC with the maximum activity on inulin was proposed for fermentation of artichoke polyfructosides. Molecular genetic techniques were used to prove that inulin hydrolysis in strain JZIC (CGMCC AS.3878) was performed by β-fructosidase of the *SUC2* gene, an enzyme hydrolyzing polyfructosides with 20 fructose residues. Changes in the primary structure of invertase of the JZIC strain, if compared to the invertase of a reference strain S288c, did not touch upon the substrate-binding and catalytic domains. The authors concluded that the specific activity of invertase of strain JZIC was not associated with the changes in the *SUC2* gene, but rather depended on glycosylation of the enzyme and/or occurred at the level of regulation of gene expression. Molecular mechanisms of inulin hydrolysis in strain JZIC remained unclear. Another strain, L610, which, in contrast to known strains, was capable of complete inulin utilization, was discovered in China [6]. Ethanol yield upon fermentation of artichoke polyfructosides by the strain was 40.0 g/L.

To summarize, it may be concluded that none of the works [1–6] contains proof that the authors dealt with new inulinase activity. Taking into account the literature data on regulation of β-fructosidase synthesis, we think that in the works [1–6], regulatory (induced, spontaneous, or natural) mutations led to overexpression of invertase providing for the yeast growth on inulin. Another, less probable mechanism providing for the yeast adaptation to inulin are mutational changes in the invertase as such, resulting in its higher affinity to inulin. This happened in the case of emergence of maltase activity in isomaltase resulting from a single amino acid replacement [39, 40]. However, the sizes of the disaccharide maltose and the polysaccharide inulin are incomparable, and, according to their sequences, the homology between true inulinase and invertase is too low (~70%) [16]. To conclude, let us point out the promising use of a food and forage yeast *Candida utilis* [41] as a overproducer of constitutive invertase that obviously should have the "inulinase activity".

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