Whey for Bioethanol Production by Yeast

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Concerns about fossil fuel depletion and the environmental effects of greenhouse gas emissions have led to widespread fermentation-based production of bioethanol from corn starch or sugarcane. However, competition for arable land with food production has led to the extensive investigation of lignocellulosic sources and waste products of the food industry as alternative sources of fermentable sugars. In particular, whey, a lactose-rich, inexpensive byproduct of dairy production, is available in stable, high quantities worldwide. Therefore, whey is indeed an ideal alternative feedstock for fuel ethanol feedstock because it can provide a remarkable 6–10 million tons of lactose annually. However, despite the availability of this massive, underutilized resource, it faces challenges in being adopted at a commercial scale since S. cerevisiae, the most common fermentative species for transforming sugars to ethanol, lacks the enzyme required for lactose utilization and thus cannot ferment whey into ethanol without further metabolic engineering.

whey bioethanol lactose S. cerevisiae Kluyveromyces

1. The Lactose/Galactose Metabolic Pathway and Its Regulation in Fungi

1.1. Lactose/Galactose Consumption in Fungi

Lactose is a disaccharide formed from galactose and glucose and chemically defined as O-ß-d-galactopyranosyl-(1–4)-β-d-glucose. In addition to lactic acid bacteria and enterobacteria that are able to utilize lactose as a sole carbon through different transport systems [1][2][3][4], some fungi, such as *Aspergillus* [5][6][7], *Trichoderma reesei* [8][9], and members of the yeast genus *Kluyveromyces* [10][11][12] also display this metabolic capability. In these fungi, lactose is utilized through two principal mechanisms: (1) the lactose is extracellularly hydrolyzed into d-glucose and d-galactose, which are subsequently taken up by the fungi and (2) the disaccharide is first imported into the cytosol and then hydrolyzed into glucose and galactose.

T. reesei uses the former pathway for lactose uptake, although the extracellular β-galactosidase (encoded by *bga1* gene) is a critical factor for lactose-induced cellulase production [13][14]. *A. nidulans* and *K. lactis*, a model microorganism of *Kluyveromyces* yeast, both utilize lactose through the latter strategy by lactose permease (encoded by *LAC12* gene)-mediated transport of the disaccharide into the intracellular compartment and subsequent hydrolysis by β-galactosidase (encoded by the *LAC4* gene) into glucose and β-d-galactose. In most eukaryotes, α-d-galactose is converted by aldose 1-epimerase to the α-anomer before entering the Leloir pathway[15][16], although filamentous fungi contain a second pathway, the oxido-reductive catabolic pathway, for α-d-galactose catabolism [6][17]. However, some strains of *K. marxianus* have been found to hydrolyze lactose outside of the cell[18][19].

Unlike *K. lactis*, *S. cerevisiae* lacks the gene for β-galactosidase synthesis, although some *S. cerevisiae* strains can secrete intracellular α-galactosidase, encoded by the *MEL1* gene. These *S. cerevisiae* strains can thus utilize melibiose as a sole carbon source [20][21]. In *S. cerevisiae* and *K. lactis*, galactose is catabolized through the Leloir pathway, which includes a four-step enzymatic reaction [15][16]. In *S. cerevisiae*, the galactokinase Gal1 mediates the initial phosphorylation of galactose in an ATP-dependent manner, resulting in galactose-1-phosphate. Then, uridine diphosphoglucose 4-epimerase (Gal10) exchanges glucose in UDP-glucose with the phosphorylated galactose, thereby generating UDP-galactose, changing the stereochemistry at C4 to create UDP-glucose. In the third step, galactose-1-phosphate uridylyltransferase (Gal7) uses glucose moieties released in the previous step to generate glucose-1-phosphate from galactose-1-phosphate. In the fourth step, glucose-1-phosphate is converted into glucose-6-phosphate through phosphoglucomutase (Gal5) activity. In *S. cerevisiae*, phosphoglucomutase is encoded by *PGM2*[16][22][23][24]. Among the four catalytic enzymes required for the Leloir pathway, Gal1, Gal7, and Gal10, which form a cluster of similarly regulated genes located on chromosome II, are specific to this pathway, while phosphoglucomutase contributes to metabolic pathways for many different carbon sources.

1.2. GAL Gene Regulation in S. cerevisiae and K. lactis

In yeast, the expression of *GAL* genes is tightly regulated by different carbon sources through three main *GAL*-specific regulatory proteins: a transcriptional activator, Gal4; a repressor, Gal80; and a ligand sensor, Gal3. Furthermore, *GAL* regulation is divided into three states depending on the carbon source. First, in the presence of aerobic or respiratory carbon sources, such as glycerol or raffinose, *GAL* genes are in an inactive or non-induced state that is expressed only at basal levels. In *K. lactis*, basal *GAL* gene expression is higher than that in *S. cerevisiae* due to the higher endogenous levels of KlGal4 protein induced by an autoregulatory positive feedback loop [25][26][27][28].

In the second state, when galactose is present in the medium, *GAL* genes are in the induced or activated state. Galactose is imported into the cytosol by Gal2 permease, where it binds Gal3p, which also allosterically binds ATP, resulting in Gal3p activation. The activated galactose-ATP-Gal3 complex interacts with Gal80 through a cooperative network of hydrogen bonds [29][30]. As a result of this interaction, Gal80 disassociates from Gal4, and Gal4 is released to transcriptionally activate *GAL* genes, ultimately leading to >1000-fold increases in *GAL2*, *GAL1*, *GAL7*, and *GAL10* expression in *S. cerevisiae* [31]. Apart from *GAL4* itself, the other *GAL* genes harbor specific upstream activating sequences (UAS) in their promoter regions that are recognized by Gal4 homodimers to induce their transcription. Notably, the *GAL4* gene lacks a Gal4 binding site in *S. cerevisiae*, while the *LAC9* gene (i.e., *KlGAL4*) in *K. lactis* has a weak UAS bind site. Consequently, Gal4 concentration in *K. lactis* is two- to three-fold higher than that in *S. cerevisiae* in the non-induced state [32][33]. In addition, there is no *GAL3* gene in *K. lactis*, and its function is substituted by Gal1, a bi-functional protein that acts as a *GAL* gene inducer in addition to its function as a galactokinase [33].

In the third regulatory state, *GAL* gene expression is actively repressed in the presence of glucose, or so-called glucose repression. To induce glucose repression, the concentration of the transcriptional repressor Mig1, a Cys2-His2 Zinc-finger DNA-binding protein, increases within minutes of yeast cell exposure to glucose, after which Gal80 enters the nucleus and interacts with the general co-repressor complex Cyc8/Ssn6-Tup1, to form a complex that targets a specific upstream repression sequence (URSG) present in the promoter region of *GAL* genes [34][35][36]. For *K. lactis*, which is more adapted to lactose/galactose-rich environments, glucose repression of *GAL*/*LAC* gene expression is absent in some *K. lactis* strains, and in strains that do exhibit glucose repression, the repression is less pronounced than in *S. cerevisiae*. This phenomenon is reflected by the fact that only the *GAL1* gene promoter in *K. lactis* carries a URSG, whereas the promoters of *GAL1*/*2*/*3*/*4* all harbor URSG motifs in *S. cerevisiae* [26][28].

In addition to this canonical model of *GAL* gene regulation, previously unrecognized regulators of *GAL* gene function are still emerging. For instance, deletion of the cytochrome c oxidase subunit *COX9* results in a respiration-deficient strain that can rapidly and efficiently ferment galactose [37]. Other work has shown that the *SIP1* gene, which encodes a component of the Snf1 kinase heterotrimer complex, is a regulator of preferential glucose consumption in *S. cerevisiae* [38]. Deletion of *SIP1* can destroy the glucose repression at 1:10 ratio of galactose-to-glucose [39]. In addition, the MIG1-related protein Imp2p has also been reported to control *GAL* gene expression by positively affecting glucose de-repression of maltose, galactose, and raffinose metabolic pathways. This protein was also found to contribute to thermal, oxidative, or osmotic stress resistances in yeast [35].

2. Bioethanol Production from Whey/Lactose by *Kluyveromyces*

While the fermentation of alcohol from cheese whey or whey permeate represents a relatively new approach to mitigating waste from the dairy industry, yeast-based fermentation of lactose from whey to generate ethanol is first introduced as early as the 1940s, or possibly earlier [40][41][42]. The separation of whey proteins generates whey permeate, which contains the majority of lactose and other whey solids. Industrial facilities that use whey permeate for ethanol production have been established in Ireland, New Zealand, the United States, and Germany. Among these, the first plant to commercially produce ethanol from whey permeate was built in 1978 in Ireland by Carbery Milk Products Ltd. to produce alcohol for beverages. This company has also produced ethanol from whey for E85 and E5 oil blends since 2005 [43]. The process first established at this plant is also employed at fermentation facilities in the United States and New Zealand.

The private producer of ethanol from casein whey feedstock is Anchor Ethanol Ltd., a subsidiary of the Fonterra New Zealand dairy cooperative, which claims to produce ~5 million gallons of ethanol annually. In the United States, whey permeate is fermented at two industrial-scale plants, both using the Carbery process, that are together responsible for 8 million gallons of fuel ethanol annually [44]. The Carbery process uses batch fermentations and continuous distillation in which the pH of the permeate is reduced to 5.0 with sour whey or acid, then pasteurized by heating to 85 °C for 15 s. The pasteurized permeate is then chilled to 34 °C, loaded into the bioreactor, and inoculated with *K. marxianus*. The conditions have been optimized for efficient and rapid lactose conversion to ethanol in 12 h fermentations, with an additional 6 h of cooling in the chamber before distillation [45].

More recently, productivity has been increased through continuous fermentation and use of whey concentrate as feedstock.

Now, the *Kluyveromyces* yeast can mediate lactose fermentation into ethanol because they harbor genes for both lactose permease and β-galactosidase. Among these species, the physiological and molecular characteristics of *K. lactis* have been widely studied as a model for "non-conventional yeasts" in comparative analyses with *S. cerevisiae* [46][47]. *K. lactis* has been used as a progenitor for other strains due to its ability to efficiently utilize the lactose in concentrated cheese whey permeate as a raw material for producing ethanol in static cultures [10]. Apart from applications in ethanol production, *K. lactis* strains are commonly used to produce β-galactosidase [48][49][50][51]. However, this species is generally considered suitable for industrial applications requiring high production and secretion of metabolites or heterologously expressed proteins in a Crabtree-negative dependent manner [52][53][54][55][56][57].

K. marxianus, a closely related species to *K. lactis*, has been adopted by several industries due to some useful features that are absent in *K. lactis* [58][59]. Like *S. cerevisae*, *K. marxianus* is a respiro-fermentative yeast that can generate energy either via oxidative phosphorylation and the TCA cycle or by fermentation to ethanol. Thus, *K. marixianus* is frequently used for ethanol production. Several studies have sought to optimize the utilization of lactose in deproteinized whey, cheese whey powder (CWP), cheese whey permeate, and cheese whey in batch and/or continuous mode fermentations [60][61][62][63][64][65][66]. In addition, *K. marixianus* can grow and ferment at elevated temperatures, enabling cost savings in ethanol production bioprocesses [67][68]. Notably, some strains of *K. marixianus* are reported to be highly thermotolerant, growing at 43 °C under aerobic conditions with lactose and/or whey permeate as the sole carbon source [67][69]. However, *K. marxianus* is characteristically Crabtree-negative, and its ethanol yields are typically lower than those of *S. cerevisiae* [69][70].

3. Strategies for Conferring Lactose Utilization to *S. cerevisiae*

3.1. Pre-Hydrolysis of Extracellular Lactose for S. cerevisiae Utilization

Lactose hydrolysis in whey fermentation results in a mixture of glucose and galactose, which can then be taken up by *S. cerevisiae* and fermented into ethanol. Three approaches to extracellular lactose hydrolysis have been established:

First, β-galactosidase can be used to hydrolyze lactose into galactose and glucose, which can then be utilized for ethanol production [71]. However, the presence of glucose induces feedback repression of galactose utilization in *S. cerevisiae*, resulting in assimilation of the produced ethanol, lower biomass, and diauxic growth.

Second, β-galactosidase and/or *S. cerevisiae* cells can be immobilized to produce ethanol [72][73][74]. Through immobilization, the cell densities are higher and thus easier to clear from the medium, thereby reducing the cost of removing cells before distillation. Immobilization can also facilitate simplification of the fermentation process, thus further saving equipment and operating costs. Similarly, strains of *S. cerevisiae* have been developed to ferment a wider range of substrates for combined fermentations, such as whey mixed with cellulosic biomass, by engineering the expression of recombinant cellulolytic proteins anchored or immobilized on the extracellular surface of the plasma membrane along with β-galactosidase [75]. Substantial research efforts have also been committed to developing the substrate for enzyme or cell immobilization and surfactants for improving enzymatic activity, such as silicon dioxide-based nanoparticles, magnetic polysiloxane–polyvinyl alcohol (mPOS–PVA), polymeric supports, and Triton X-100 [76][77][78]. However, immobilization strategies are accompanied by some disadvantages, especially catabolite repression by glucose after lactose hydrolysis and subsequent diauxic growth of the yeast. This lag in lactose fermentation following glucose depletion extends the fermentation time and increases the cost of fermentation.

Third, co-immobilization or co-culture of *S. cerevisiae* with other microorganisms, which secrete extracellular β-galactosidase, can facilitate lactose fermentation by *S. cerevisiae*, thus enhancing ethanol yields and shortening fermentation time. Co-immobilization of the two yeasts has been demonstrated to increase the percentage of theoretical yield over that of monocultures and enhance the overall volumetric productivity. In addition, immobilized co-cultures are reportedly more effective than suspension cultures for high-temperature ethanol fermentations [79][80][81][82][83][84].

3.2. Protoplast Fusions of S. cerevisiae and Kluyveromyces *spp.*

Protoplast fusion, a part of evolutionary engineering, has a great potential for genetic analysis and strain improvement. It breaks down the barriers to genetic exchange imposed by conventional mating systems. It can serve the purpose of developing a strain with mixed substrate fermentation abilities [85][86][87]. This technique is generally applied for developing inter specific, intra specific and inter generic, intra generic supra hybrids with higher capability. It is a significant tool for genetic manipulation as it resolves the barrier to genetic exchange imposed by conventional mating systems. It is particularly useful for industrially important microorganisms [87]. Although *S. cerevisiae* cannot utilize lactose, it is tolerant of ethanol and exhibits higher productivity than other fermentation species, making it the most reliable option for ethanol production. In order to enhance production by combining the characteristics of ethanol tolerance and lactose utilization in a single strain, hybrid strains of *S. cerevisiae* and *Kluyveromyces* spp. can be generated through protoplast fusion [88][89][90]. For example, Guo et al. obtained a stable hybrid of *K. marxianus* with an *S. cerevisiae*, showing higher lactose utilization rates and ethanol productivity than the parent strain [89]. Krishnamoorthy and colleagues constructed a hybrid using a temperature-tolerant *S. cerevisiae* with *K. marxianus* that resulted in a 12.5% increase in ethanol productivity at 42 °C [88]. Similarly, Xin generated an *S. cerevisiae–K. marxianus* fusion strain with higher lactose fermentation rates and ethanol tolerance than that of the *K. marxianus* parental strain, most likely due to its elevated production of linoleic acid and other unsaturated fatty acids [91]. It should be noted that hybridization can increase the amount of chromosomal DNA in each cell [88][89].

3.3. Exogenous Expression of the Lactose Hydrolase Gene in S. cerevisiae

One robust approach for increasing the range of sugar substrates for *S. cerevisiae* fermentation is through the transgenic expression of lactose hydrolysis enzymes from other lactose-consuming microbes. *S. cerevisiae* can thus be metabolically engineered to consume lactose through amplification, cloning, and the introduction of naturally occurring genes or pathways that mediate its uptake and catabolism. The majority of lactose utilization genes used in yeast have been obtained from either *Escherichia coli*, *Kluyveromyces* species, or the filamentous fungus *A. niger*.

3.3.1. Lactose Metabolism Genes from *E. coli*

In *E. coli*, three genes are required for the uptake and metabolism of lactose and related sugars, including *lacZ*, *lacY*, and *lacA* [92][93][94]. *lacZ* encodes β-galactosidase, which converts lactose into allolactose and subsequent metabolic intermediates. *LacY* encodes the lactose permease (LacY) transporter, which mediates lactose uptake into the cell. In order to ensure that *S. cerevisiae*, a eukaryote, can successfully express the prokaryotic *lacZ*, the yeast promoter *CYC1* is typically fused to *lacZ* prior to its transformation into yeast. Since *lacZ* expression is repressed by glucose, an additional ~300 nucleotide DNA fragment is also fused to the expression construct upstream of *CYC1* to drive β-galactosidase expression in the presence of glucose in *S. cerevisiae* [95]. However, although *lacZ* expression was not repressed by glucose in these yeast transformants harboring *CYC1*-*lacZ*, they were still unable to utilize lactose because they lacked the *E. coli* lactose transport system and therefore could not import lactose to the cytosol for access by β-galactosidase.

To overcome this obstacle, researchers sought to engineer the secretion of β-galactosidase into the culture medium. Porro and his coworkers constructed a lactose-consuming *S. cerevisiae* strain that overexpressed the *lacZ* gene from *E. coli* and then relied on lysis of the older mother cells to release the recombinant β-galactosidase. Cell lysis was induced by overexpression of the transcriptional activator *GAL4*. Characterization of the fermentation properties of the transformed yeast strains indicated that β-galactosidase released in cell lysates enabled growth on lactose as a sole carbon source or in medium containing whey as growth substrate. Furthermore, these strains were found to efficiently produce ethanol during the stationary culture phase following growth on lactose medium [96]. Martegani and coworkers found that cell lysis was independent of heterologous *LacZ* expression in the mother cells [97]. As suggested above, *GAL4* overexpression was proposed as the causative factor in mother cell lysis since the high accumulation of Gal4p can alter regulatory pathways that affect the composition and structural integrity of cell walls, ultimately leading to lysis in older cells. In addition, Gal4p participates in the repression of galactose utilization in the presence of glucose, and excess Gal4p levels have been shown to possibly mediate concurrent glucose and galactose consumption in the *lacZ*-expressing yeast.

3.3.2. The Lactose Metabolism Genes from *A. niger*

A. niger can utilize an exceptionally broad spectrum of carbon substrates and is known to secrete numerous glycoproteins, including β-galactosidase, which enables hydrolysis of lactose in acid whey [98]. A lactose-consuming *S. cerevisiae* strain was constructed through the expression of *A. niger* cDNA encoding secreted β-galactosidase. The *lacA* gene (which encodes β-galactosidase in *A. niger*), including its N-terminal signal sequence that directed its extracellular secretion, was inserted between the alcohol dehydrogenase (*ADH1*) promoter and a transcriptional terminator to construct the pVK1.1 vector construct [99]. The pVK1.1 plasmid was then transformed into *S. cerevisiae* resulting in β-galactosidase overexpression. Although the transformant could hydrolyze lactose, the generation time was 8 h in lactose minimal medium. However, the plasmid exhibited high stability, and 84% of the cells still contained the plasmid after nine doublings on whey permeate medium [99]. Ramakrishnan studied the fermentation properties of the transformants, GRF167(pVK1.1). The transformant can hydrolyze the lactose in the medium, and the glucose and galactose from lactose can be utilized at the same time [100]. In addition, a polyploidy distiller's yeast transformed the same plasmid to obtain a lactose-consuming strain, and the transformants display a higher lactose hydrolysis rate with a simultaneous uptake of glucose and galactose. In addition, the ethanol yield of the transformants is 90.16% the theoretical yield in lactose YP medium; however, the stability of the plasmid is poor, as, during fermentation, only about 10% of the cells retain the plasmid [100].

A lactose-consuming flocculent brewer's yeast, W204-FLO1L(INT)/pLD1, was constructed through heterologous expression of *lacA* on the pVK1.1 plasmid, the *CUP1* copper resistance marker used to screen successful transformants. The transgenic yeast cells were able to use lactose with no obvious impact on flocculability [101]. Domingues and coworkers used a flocculent uracil auxotrophy mutant of *S. cerevisiae* carrying pVK1.1 as a parent strain to construct a flocculent lactose-consuming strain, NCYC869-A3/pVK1.1 [102]. The recombinant yeast exhibited high extracellular β-galactosidase activity. Although galactose uptake was at least partially repressed by glucose in this strain, the strain showed a lactose uptake rate close to 1.7 g/L/h and with ethanol contents close to the maximum theoretical yield [102].

3.3.3. The Lactose Metabolism Genes from *Kluyveromyces* spp.

Different from *A. niger*, which breaks down lactose in the extracellular space, yeast in the genus *Kluyveromyces* transports lactose into the cell for metabolism. Therefore, a lactose permease is needed to access the substrate in addition to the lactose hydrolase. The lactose hydrolase, i.e., β-galactosidase, is encoded by the *LAC4* gene, while the lactose permease is encoded by the *LAC12* gene. Since *Kluyveromyces* is relatively close to *S. cerevisiae*, phylogenetically, the eukaryotic lactose transport system from *Kluyveromyces* is more compatible with *S. cerevisiae* regulatory mechanisms. Thus, *S. cerevisiae* can be engineered to grow on lactose through the simultaneous expression of lactose permease and non-secreted β-galactosidase from *Kluyveromyces*.

Sreekrishna was the first to construct Lac+ *S. cerevisiae* strains using a shuttle vector carrying a 13 kb region of the *K. lactis* genome [103]. The 13 kb region contained *LAC4* and a flanking upstream sequence from *K. lactis*. Despite the low lactose uptake rate in transformants, these uptake assays confirmed that the lactose permease gene, *LAC12*, was contained in that flanking sequence from *K. lactis*, between 2.0 and 8.6 kb upstream of *LAC4*. That study represented the first report of heterologous expression of a eukaryotic membrane-bound permease, which laid a foundation for the subsequent cloning of lactose permease and lactose hydrolase genes from *K. lactis* [103]. In order to improve the lactose uptake rate and increase the mitotic stability of the heterologous construct, researchers then created the MRY276 yeast strain by inserting the *LAC4* and *LAC12* genes from *K. lactis*, under the control of the *CYC1-GAL* galactose-inducible promoter, into the *RDN1* (ribosomal DNA) locus [104]. MRY276, which harbored multiple copies of both *LAC4* and *LAC12*, retained the Lac+ phenotype for longer than 60 generations in growth on non-selective medium, although it exhibited slow growth. The suboptimal growth characteristics of this strain were likely attributable to the burden imposed by overexpression of these transgenes. To resolve this issue, transgenic MRY276 was crossed with wild-type strains to yield meiotic segregants with faster growth and the capacity for lactose assimilation, such as the diploid strain MRY286 [104]. However, this strain showed low ethanol production but high biomass production on lactose minimal medium.

Domingues constructed the Lac+ flocculent *S. cerevisiae* strain, T1, that expressed *LAC4* and *LAC12* from *K. marxianus* rather than *K. lactis* [105]. The T1 strain showed a lower capacity for flocculation than that of the parent strain but showed a doubling time of 5 h, which was substantially faster than the 6.7 h reported for the original Lac+ strain constructed by Sreekrishna and Dickson [103], but slower than the *K. marxianus* donor of the *LAC4* and *LAC12* genes. However, after an adaptation period, where the strain was kept in liquid lactose medium, refreshed periodically, fermentation assays indicated that T1 could metabolize ~90 g/l lactose in liquid medium, thus highlighting increases in rates of both biomass accumulation and ethanol production from lactose.

In general, the use of a lactose-consuming strain for direct fermentation of whey to produce ethanol is not economically feasible because the low lactose content in whey results in low ethanol titer (i.e., 2–3% *v*/*v*). Therefore, fermentation should begin with concentrated whey to obtain high ethanol yields at the end of the process, which also requires high tolerance to ethanol and osmotic pressure. In *S. cerevisiae*, the accumulation of trehalose is widely considered a critical determinant for the improvement of stress tolerance in yeast, and the deletion of the trehalose degrading enzyme gene can significantly increase intracellular trehalose content [106].

In particular, a neutral cytosolic trehalase (*NTH1*) and an acidic vacuolar trehalase (*ATH1*) have been identified as the main trehalose hydrolases in *S. cerevisiae* [107][108]. In order to obtain a Lac+ *S. cerevisiae* strain that tolerates high sugar and ethanol conditions associated with concentrated whey fermentation, the Δ*ath*Δ*nth* Lac+ strain AY-51024A was generated, which expressed the permease and β-galactosidase genes from *K. marxianus* [109]. In this strain, the *ATH1* and *NTH1* loci were used as the target regions for integrating the *LAC4* and *LAC12* genes driven by the *PGK1* promoter. AY-51024A showed roughly equal β-galactosidase activity during growth on different carbon sources and exhibited high tolerance to ethanol, and could withstand higher osmotic pressure than the parent strain. In addition, it showed an almost equal rate of glucose uptake and a higher rate of galactose uptake than its parent strain, AY-5. However, this transgenic strain was subject to glucose repression, and lactose uptake rates were very slow in lactose medium (0.98 g lactose/g cell/h). Further analysis confirmed that glucose repression of lactose/galactose metabolism was responsible for the slower lactose uptake rather than the result of low copy number of *LAC4* and *LAC12* [109].

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