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Improving the Utilization of Isomaltose and Panose by Lager Yeast *Saccharomyces pastorianus*

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Abstract: Approximately 25% of all carbohydrates in industrial worts are poorly, if at all, fermented by brewing yeast. This includes dextrins, β-glucans, arabinose, xylose, disaccharides such as isomaltose, nigerose, kojibiose, and trisaccharides such as panose and isopanose. As the efficient utilization of carbohydrates during the wort's fermentation impacts the alcohol yield and the organoleptic traits of the product, developing brewing strains with enhanced abilities to ferment subsets of these sugars is highly desirable. In this study, we developed *Saccharomyces pastorianus* laboratory yeast strains with a superior capacity to grow on isomaltose and panose. First, we designed a plasmid toolbox for the stable integration of genes into lager strains. Next, we used the toolbox to elevate the levels of the α-glucoside transporter Agt1 and the major isomaltase Ima1. This was achieved by integrating synthetic *AGT1* and *IMA1* genes under the control of strong constitutive promoters into defined genomic sites. As a result, strains carrying both genes showed a superior capacity to grow on panose and isomaltose, indicating that Ima1 and Agt1 act in synergy to consume these sugars. Our study suggests that non-GMO strategies aiming to develop strains with improved isomaltose and panose utilization could include identifying strains that overexpress *AGT1* and *IMA1*.

Keywords: panose; isomaltose; wort; lager; *Saccharomyces pastorianus*

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1. Introduction

Lager is the most popular type of beer and represents more than 90% of the total production in the world [1]. This beer type is fermented by strains of the lager yeast, *Saccharomyces pastorianus*, the origin of which derives from the domesticated allotetraploid interspecies hybrid between *Saccharomyces cerevisiae* and the cold-tolerant *Saccharomyces eubayanus* [2–5]. The recent isolation of an *S. eubayanus* strain [6], and its full genome sequence [7], has opened the door for the generation of novel lager yeast hybrids. Besides, new or genetically improved lager strains with novel or superior features, such as increased alcohol production, new flavor spectra, reduced amounts of off-flavors, are always in demand to improve production efficiency and to accommodate new consumer trends [8–11].

Saccharomyces pastorianus lager yeast are historically divided into two lineages: Saaz-type (group I) and Frohberg-type (group II) [12,13]. More recently, [14] have proposed a new group of lager yeast, group III, to be considered in the existing classification. The Saaz-type lager strain Unterhefe No. 1, later termed *Saccharomyces carlsbergensis* and then *S. pastorianus*, was the first pure culture of yeast. This strain was isolated by Emil C. Hansen in 1883 [15] at Carlsberg Research Laboratory and recently re-isolated from an old beer bottle from

the Carlsberg Museum beer collection [13]. The Saaz-type lager yeast is better adapted to cold fermentation temperatures than the Frohberg-type yeast. However, under current industrial brewing conditions, Frohberg-type lager yeast, for example, Weihenstephan strain WS34/70, provide faster fermentation and better utilization of the wort sugars, also called attenuation. A crucial difference between these two lineages is that the Saaz-type lager yeast lacks specific fragments of the *S. cerevisiae* parental genome [12,13].

The production of beer relies on yeast for the efficient fermentation of starch hydrolysate (wort) to produce CO_2, ethanol, and many other secondary products that provide specific aromas and flavors [16]. Approximately 90% of the solids in wort are carbohydrates. Glucose, fructose, sucrose, maltose, and maltotriose are commonly regarded as being fermentable sugars. However, nearly 25% of all carbohydrates are unfermentable; these include dextrins, arabinose, xylose, maltulose, ribose, disaccharides such as isomaltose, nigerose, kojibiose, trisaccharides such as maltotriulose, panose, and isopanose, and β-glucans [17–26]. During beer fermentation *S. cerevisiae* and *S. carlsbergensis* have a general preference to utilize sucrose and hexoses first, such as glucose and fructose, followed by maltose and maltotriose [18]. Commonly, the residual sugars are maltotrioses, panose (6-α-glucosyl maltose), isopanose (6-α-maltosyl-glucose) [27,28], and isomaltose [19]. Importantly, the wort composition and the efficient utilization of the carbohydrates by yeast has a direct influence on the efficiency of the process and the organoleptic profile of the final product [29–32]. Control of the utilization of these sugars is therefore highly desirable.

In the early 1970s, [19] reported efficient attenuation of some of the low abundance sugars in wort by *S. cerevisiae* ale brewing strains, including the complete or partial attenuation of isomaltose and incomplete or null utilization of panose. Moreover, we previously made new *S. pastorianus* Frohberg-type lager yeast hybrid strains and selected the ones exhibiting an improved isomaltose and panose utilization [33]. Interestingly, the transcription levels of several genes in these hybrids were altered when their performances were evaluated under real brewing conditions. Of particular interest were significant increases in the transcription levels of two genes, *IMA1* and *AGT1,* which could potentially influence the wort's attenuation.

IMA1 and *AGT1* are located in the *MAL* loci together with more genes required for efficient di- and trisaccharide utilization by *Saccharomyces* yeast [34–38]. *IMA1* encodes the major isomaltase [35,36], and *AGT1* encodes a broad substrate specificity transporter with specificities that include maltose and maltotriose [22,35,39–41]. In *Saccharomyces* species, the genes required for maltose utilization are organized in the *MAL* loci. Typically, *MAL* loci are located in the subtelomeric regions and have been found in up to five chromosomes [5]. Each locus comprises a complex arrangement of genes including three essential gene families: *MALT*, *MALS*, and *MALR*. The *MALT* gene family, including *AGT1*, encodes proton symporters. However, amongst these transporters Agt1 is the only one that is capable of transporting maltotriose [22]. The *MALS* gene family, including *IMA1*, encodes glucosidases [35,42]. The *MALR* gene family encodes the regulators required to activate the expression of *MALT* and *MALS* [35,39,43,44].

This study aimed to examine the role of *AGT1* and *IMA1* in the consumption of sugars, which commonly remain unused during the beer brewing process by lager yeast, by a genetic approach. First, to facilitate a stable gene integration, we designed a plasmid toolbox suitable for *ura3* lager yeast strains. This toolbox facilitates gene integration into defined genomic loci of two *S. pastorianus* laboratory strains. Second, using the toolbox, we next integrated synthetic *IMA1* and *AGT1* genes under the control of strong constitutive promoters. Lastly, we evaluated the effect of the additional gene copies of *IMA1* and *AGT1* on the growth of several lager yeast when employing glucose, panose, or isomaltose as the sole carbon source.

2. Materials and Methods

2.1. Strains and Cultivation Methods

The yeast strains used in this study are listed in Table 1. Model *S. pastorianus* strains LG160 and LG230, which exhibited stable mating types, were generated by Lise Hoffman at the Carlsberg Research Laboratory [45]. Both model strains are mutants of *S. carlsbergensis* C80-CG65 and *S. carlsbergensis* C80-CG110 [46], meiotic segregates of *S. carlsbergensis* 244. LG160 is a mutant strain derived from the diploid strain C80-CG65, and LG230 is a meiotic segregate of the hybrid C80-CG65 and C80-CG110. For routine maintenance of yeast, YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) agar plates were used. For growth on plates 20 g/L agar was used. For genetic transformations using auxotrophic markers, synthetic completed (SC) and drop-out media (SC-URA) were used. All the media used contained 2% glucose, pH 5.8. All agar plates were incubated at 25 °C for the required time.

Table 1. Strains used in this study.

Strain	Parental Strain	Integration Site	Genotype	Source
LG160	N.A.	N.A.	*S. pastorianus* (MATa/MATa ura3 ura3)	Carlsberg collection
LG230	N.A.	N.A.	*S. pastorianus* (MATalpha/MATalpha ura3-ca-Δ2 ura3-KpnI met2-ca-Δ)	Carlsberg collection
sCP034	LG230	XI-I	P_{CCW12} -> *IMA1*	This study
sCP041	LG160	XI-II	P_{PGI1} -> *AGT1*, P_{PGK1} -> *IMA1*	This study
sCP047	LG230	XI-I	P_{PGI1} -> *AGT1*, P_{PGK1} -> *IMA1*	This study
sCP101	LG160	X3	P_{PGI1} -> *AGT1*	This study
sCP107	LG230	X3	P_{PGI1} -> *AGT1*	This study

For the micro fermentations, seed cultures were grown overnight in YPD liquid media. Then 3µL of the overnight culture was inoculated into 100 µL culture of defined media (DM) which included synthetic complete media YNB (6.7 g/L Difco YNB without amino acids; Becton, Dickinson and Company, Sparks, MD, USA) supplemented with ammonium sulfate, 2 g/L SC (Synthetic Complete Supplement Mixture, HiMedia Laboratories Private Limited, Mumbai, India), the carbon source, and buffered with potassium hydrogen phthalate to pH 5.5 [47]. Cell growth was followed for 120 h by measuring the optical density at 600 nm with continuous agitation set at maximum amplitude and maximum speed and incubated at 20 °C using a Bioscreen C MBR (Oy Growth Curves Ab Ltd., Turku, Finland) [48,49].

Escherichia coli DH5α cells were used as a host for plasmid cloning and propagation. *E. coli* DH5α cells were grown on lysogeny broth (LB) medium supplemented with 100 mg/L of ampicillin.

2.2. Illumina and PacBio Whole Genome Sequencing and Assembly

Genomic DNA of lager yeast LG160 and LG230 was isolated using the Quick-DNA Fungal/Bacterial Miniprep Kit from ZYMO research (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's recommendations. Then, their genomes were sequenced using the Illumina HiSeq (Hayward, CA, USA) sequencing platform in order to obtain paired-end reads (Figure 1a). Following this, the assembly of the LG160 genome was performed using ABySS [50] software. The assembled genome was further polished using Pilon [51] software, and its quality was finally estimated with QUAST [52]. Importantly, only contigs with sizes exceeding 500 base pairs (bp) were considered for the final genome assembly and to calculate the summary statistics. LG230's genome was assembled differently. Besides using the Illumina HiSeq sequencing platform, it was also sequenced using the Pacific Bioscience (PacBio, Menlo Park, CA, USA) sequencing platform. The long reads, product of the PacBio sequencing platform, were fed to Canu [53] software to produce the first draft of the assembly of the LG230 genome. Next, to enhance the quality of the assembly, the Illumina paired end reads of LG230 were mapped against the

assembly obtained via Canu software. Similar to the assembly of the other parental strain, the assembly of the LG230 genome was polished using Pilon [51] software.

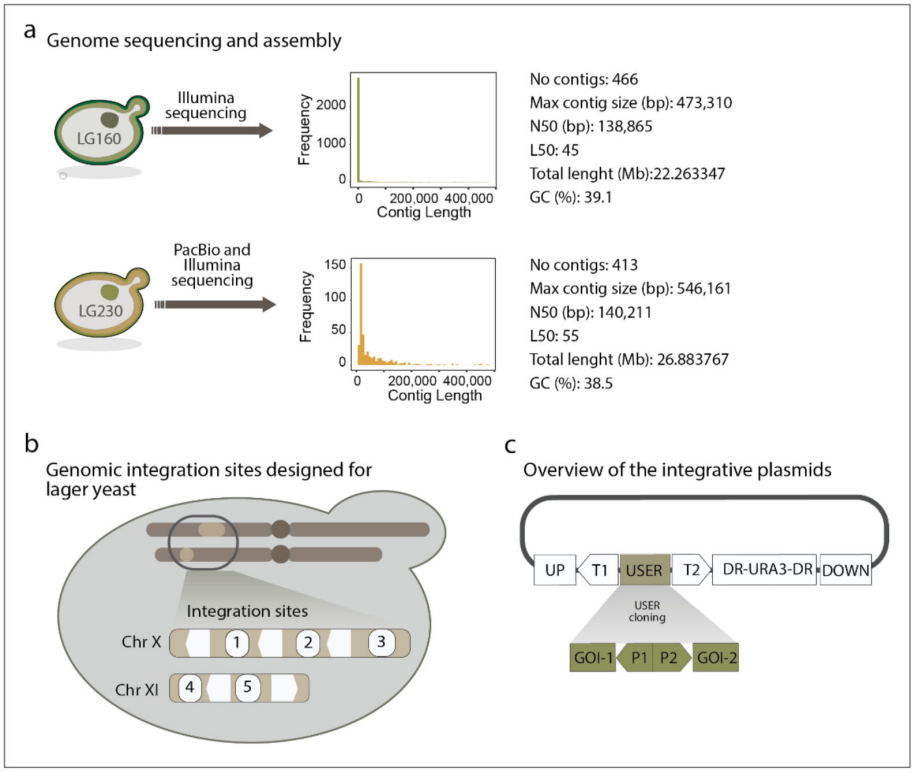

Figure 1. Overview of the genome sequencing, assembly, and design of a gene integration toolbox for *S. pastorianus* yeast. (**a**) The genomic DNA of the lager yeast LG160 and LG230 was sequenced using two different approaches. The genome of LG160 was solely sequenced by Illumina HiSeq sequencing platform and the genome of LG230 was sequenced using a mix of sequencing technologies: Illumina HiSeq and PacBio. The Quality of the final genome assembly was assessed by QUAST [52] software. The statistics of the assembly are presented as number of contigs (No contigs), maximum contig size, N50, L50, GC content, and the total length. (**b**) Five integration sites were design based on the gene integration toolbox presented in [54]. The integration sites, numbered boxes, are organized in clusters in two chromosomes which are homologous to *S. eubayanus* chromosomes X (Chr X) and *S. cerevisiae* chromosomes XI (Chr XI). (**c**) Overview of the integrative plasmid suitable *S. pastorianus* lager strains auxotrophic for uracil. The cassette containing a single or a double bidirectional promotor (P1-P2) and up to two genes of interests (GOI) can be assembled by USER^{TM} cloning into the parental vectors. The recombination sites in the parental vectors, UP and DOWN, are unique for each integration site.

2.3. Design of Integration Sites

The design of the integration sites for *S. pastorianus* (Figure 1b) was based on the integration sites toolbox suitable for *S. cerevisiae* yeast described in [54]. Although the alignment between the integration sites described in [54] and the nucleotide sequences of the homologous positions in *S. pastorianus* lager yeast showed similitude, new integration sites had to be designed. The integration sites for *S. pastorianus* yeast are among 800 and 1000 base pairs long. These are distributed in two chromosomes which are homologous to chromosomes X and XI of *S. eubayanus* and *S. cerevisiae* (see Figure S1), respectively. The full sequence of the integration sites is shown in the Table S1.

2.4. Plasmid Construction

Bio-bricks were amplified using Phusion U Hot Start DNA polymerase (Thermo Scientific, Vilnius, Lituania) under the following conditions: 98 °C for 2 min, 34 cycles of 98 °C for 10 s, 57 °C for 15 s, 72 °C for 15 s/1 kb, 72 °C for 5 min. The used primers

and templates are listed in Table S2. All the bio-bricks were gel purified and treated with Fast-Digest *DpnI* enzyme (Thermo Scientific, Vilnius, Lituania). All plasmid were constructed using uracil-specific excision reagent (USER^{TM} enzyme, New England BioLabs, Ipswich, MA, USA) [55]. The plasmids carrying the USER^{TM} cassette, pCP0005, pCP0011, and pCP0015, also called base plasmids (Figure 1c), were assembled by using the biobricks bCP001, bCP002, bCP005, bCP006, bCP009, bCP010, bCP011, and bCP012. The biobricks and plasmids details are described in Tables S3 and S4.

Base plasmids were prepared by digestion using Fast Digest *AsiSI* (Thermo Scientific, Vilnius, Lituania) enzyme followed by a nicking step using the endonuclease *Nb.BsmI* (Thermo Scientific, Vilnius, Lituania). Then, synthetic genes *AGT1* and *IMA1* were cloned into the base plasmids via USER^{TM} cloning [55]. The sequenced of the synthetic genes is provided in Table S5. The genes, arranged individually or in pairs, were preceded by a KOZAK sequence (*AAAACA*) to enhance the translation efficiency. In the cases where both genes were cloned in the same base plasmid (pCP0044 and pCP0047), these were arranged divergently and expressed under the control of strong constitutive promoters. Specifically, *AGT1* was expressed under the control of p*PGI1;* and *IMA1* was expressed under the control p*PGK1*. Synthetic genes were also expressed individually. In this scenario, *IMA1* was expressed under the control of p*CCW12* (pCP0032), and *AGT1* under the control of p*PGI1* (pCP0066).

USER^{TM} reactions were incubated at 37 °C for 20 min followed by an extra 20 min at room temperature. Furthermore, the reactions were transformed into 20 µL of chemically competent *Escherichia coli* DH5α cells with growth overnight at 37 °C on LB agar plates supplemented with 100 mg/L ampicillin. The next day, single colonies were isolated and used to verify the plasmid assembly. The correct transformants were then propagated on LB liquid media supplemented with 100 mg/L ampicillin and incubated overnight at 37 °C to generate the required biomass. Plasmid purification was performed using the GenElute Plasmid Miniprep kit by Sigma-Aldrich (Merck Life Science S.L., Madrid, Spain). Prior to integration, all the constructs were verified by Sanger sequencing. Finally, to prepare the plasmids for genome integration, they were linearized using *NotI-HF* (New England BioLabs, Ipswich, MA, USA) under the conditions recommended by the seller.

2.5. Strain Construction

Yeast transformations were performed following the Lithium-Acetate transformation protocol [56] with slight modifications. As a repair template, 150 ng of the required plasmid was used, and the heat shock was performed at 42 °C for 30 min. The transformation suspensions were inoculated on SC-URA agar plates and incubated at 25 °C for seven days. Then, single colonies were isolated on individual SC-URA agar plates in order to discard false positives. Finally, colony PCRs confirmed the correct integration of the constructs. The colony PCRs were performed using Taq 2x Master Mix (New England BioLabs, Ipswich, MA, USA) under the following conditions: 95 °C for 10 min, 29 cycles of 95 °C for 10 s, 51 °C for 15 s, 72 °C for 1 min, 72 °C for 5 min.

2.6. Phenotypic Characterization

The cell growth was followed by measuring the optical density at 600 nm with continuous agitation and incubating at 20 °C temperature using a Bioscreen C MBR (Oy Growth Curves Ab Ltd., Turku, Finland) [48,49]. R Studio version 1.4.1106 (RStudio Inc., Boston, MA, USA) [57] and the corresponding packages, including ggplot2 v.3.3.5 [58] and growthrates v0.8.2 [59], were used for the data analysis and visualization. The growth curves presented in Figure 2a represent the mean curves of at least three independent samples. For the estimation of the growth parameters lag phase, maximum growth rate (µ), and the carrying capacity (*K*), we considered only the first 120 h of growth data. This period of time might be crucial in the industrial beer brewing processes. Moreover, solely the data sets in which the fitness to the parametric growth model Baranyi–Roberts [60] was $r^2 > 0.9$ were used (see Table S6).

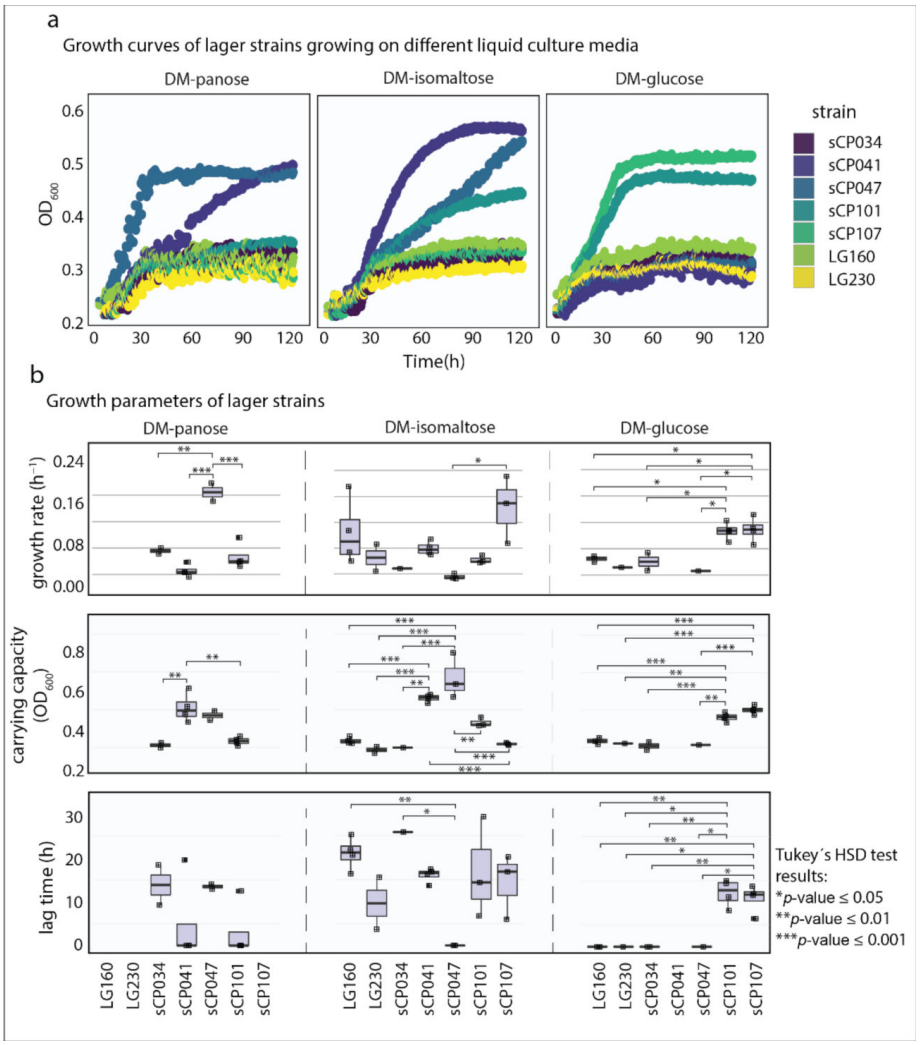

Figure 2. Phenotypic characterization of the engineered and parental lager strains on three different culture media. (**a**) The parental (LG160 and LG230) and engineered strains (sCP034, sCP041, sCP047, sCP101, and sCP107) were cultivated in three different culture media (DM-panose, DM-isomaltose, and DM-glucose) at 20 °C for five days under continuous agitation, and their growth was measured by optical density at a wavelength of 600 nm using a Bioscreen C MBR (Oy Growth Curves Ab Ltd., Turku, Finland). (**b**) The growth parameters were estimated using the growthrates v0.8.2 [59] R package, specifically by the parametric growth model Baranyi–Roberts [60]. Only fitted curves with $r^2 > 0.9$ were considered. * indicates a statistically significant difference among the mean values of the growth parameters of two samples with a *p*-value ≤ 0.05; ** indicates a *p*-value ≤ 0.01; and *** indicates a *p*-value ≤ 0.001.

2.7. Statistical Analysis

The statistical analysis was performed in R Studio version 1.4.1106 (RStudio Inc., Boston, MA, USA) [57]. Up to four replicates were used to estimate the mean values reported in this study. The number of replicates utilized to estimate the mean values of the growth parameters varied because only sets of growth data with a coefficient of determination (r^2) > 0.9 were considered. To compare the mean values of the estimated growth parameters, we performed a one-way analysis of variance (ANOVA) followed by the post hoc Tukey's HSD test when required. The comparisons were performed between the strains by grouping the samples by the culture media utilized in the growth experiments.

3. Results

3.1. Whole Genome Sequencing and Assembly of Genomes of Model Lager Yeast

The assembly of the LG160 genome, which was obtained using Illumina paired reads, produced 466 contigs, an *N50* value of 138,865 bp, and an *L50* contig count of 45. QUATS evidenced an LG160 genome size of 22.223 Mb and GC content of 39.1%. These estimates are similar to the genome assembly of the strain Weihenstephan 34/70 previously reported by different authors [13,61,62], in which the genome size varies between 22.3 Mb to 22.9 Mb, and the GC content varies among 39% to 39.1%. The genome of the parental strain LG230 was assembled differently. We obtained a high-quality assembly of the LG230 genome by using a combination of short and high-quality Illumina paired reads with PacBio long low accuracy read. The use of similar strategies has been recently reported by several research groups [5,39,63,64]. Following this approach, we generated an LG230 genome assembly of size 26.883 Mb, which is four million base pairs longer than the genome of LG160. Moreover, this assembly consists of 413 contigs, with an *N50* value of 140,211, an *L50* contig count of 55, and a GC content of 38.5%. The statistics of the assembled genomes are summarized in Figure 1a, and full details of these are listed in Table S7.

3.2. Endogenous IMA1 and AGT1 Alleles in the Model Lager Yeast

The nucleotide sequence of *S. cerevisiae AGT1* reported by [33] was used as a query to identify homologs in the two model lager genomes. This analysis uncovered two nucleotide sequences in each lager yeast homologous to *S. cerevisiae AGT1*. However, similarly for both strains, one of the homologs contains a premature stop codon terminating the protein at residue 394. As a result, the major facilitator superfamily (MFS) domain, which is essential for transporting the sugars into the cell, is disrupted. Likewise, the second potential *AGT1* homolog encodes a protein that lacks the first 40 and the last 30 residues compared to Atg1. Although the MFS domain is complete, the possibility exists that it may be functionally compromised. Nevertheless, a similar analysis could be performed considering as query non-*S. cerevisiae AGT1* homologs.

Similarly, we used the nucleotide sequence of *S. cerevisiae IMA1* reported by the same authors as a query to identify genes encoding putative isomaltases in the model lager yeast LG160 and LG230, as well as in the two available *S. eubayanus* genomes, FM1318 [7] and CBS12357 [5]. For the two *S. eubayanus* strains, FM1318 and CBS12357, this analysis uncovered four and seven genes, respectively. Similarly, in lager yeast, ten genes were identified in LG230 and six in LG160.

Then, to increase the insights into the relationship between the isomaltase variants, we constructed two phylogenetic trees [65–68], one for each model lager yeast, that included: Ima1-5, Mal32, and Mal12 from *S. cerevisiae* S288c; Ima1 variants from additional nine *S. cerevisiae* strains; and the isomaltases identified in both *S. eubayanus* strains, FM1318 [7] and CBS12357 [5]. For more details about the construction of the phylogenetic trees, please refer to supplementary material. This analysis uncovered five (hit1, hit2, hit3, hit5, hit7), and three (hit1, hit2, hit4) Ima1 homologs in *S. eubayanus* CBS12357 [5] and *S. eubayanus* FM1318 [7], respectively (see Figure S2). Also, this analysis demonstrated the presence of two Ima1 homologs in LG160 (LG160_hit1_8003 and LG160_hit2_7920), and two Ima1 homologs in LG230 (hit1_18878 and hit4_1151). Despite the majority of the Ima1 homologs found in *S. eubayanus* and lager yeast forming a new clade, these appear to be more closely related to Ima1 rather to Ima2-4 or *IMA5*.

3.3. Effect of the Integration of an Extra Copy of AGT1 and IMA1 on the Growth of Lager Yeast Saccharomyces pastorianus

We utilized the plasmid toolbox described above to integrate single copies of the synthetic genes *AGT1* and *IMA1* into the genome of the model lager yeast LG160 and LG230. The list of the yeast used in this study is shown in Table 1. Then, we evaluated the effect of the genomic integration of extra copies of *AGT1* and *IMA1* on the growth of lager yeast. To this purpose, model and engineered lager strains were grown in defined

liquid media (DM) using glucose, panose, or isomaltose as the main carbon source. The fermentations of the carbon sources were carried out at a micro-scale using 100 µL of culture media incubated under continuous agitation for five days at 20 °C using a Bioscreen C MBR (Oy Growth Curves Ab Ltd., Turku, Finland) [48,49]. The biomass formation was measured as the increment of the optical density (OD) at 600 nm (OD_{600}). Growth curves and growth parameters obtained from the phenotypic characterization are presented in Figure 2. For more details of the estimation of the growth parameters, please refer to supplementary material.

The engineered yeast sCP047 and sCP041, which have integrated both *IMA1* and *AGT1*, exhibited a superior capacity to grow on DM-panose compared to the model lager yeast LG160 and LG230. Remarkably, sCP047 displayed the greater growth rate (µ) on DM-panose (0.18 h^{-1}). The statistical analysis indicated no significant differences among µ values of the rest of the engineered strains sCP034, sCP041, and sCP101 (0.03 to 0.07 h^{-1}). Moreover, sCP041 and sCP047 reached a maximum population size, also referred to as carrying capacity (K), of OD_{600} 0.47 and 0.51, respectively. Whereas sCP101 and sCP034 showed a 30% to 40% lower K compared to sCP047 and sCP041, respectively. Interestingly, no statistical differences in the lag time values were observed among the engineered lager yeast growing on DM-panose.

sCP041 and sCP047 also exhibited a superior ability to grow on DM-isomaltose. These strains presented *K* values (OD_{600} of 0.53 to 0.80) when growing on this media which were 10% and 40% higher than when growing on DM-panose. In contrast, no significant differences were observed among the *K* values of the parental and the engineered strains sCP034, sCP101, and sCP107 (OD_{600} of 0.29 to 0.43). Our results are consistent with the study reported by [35]. According to these authors, the growth of *S. cerevisiae* in a media containing isomaltose as a carbon source requires the presence of both genes, *AGT1* and *IMA1* [35].

The engineered strain sCP047 also presented one of the shortest periods of lag phase when it was cultivated on DM-isomaltose (0.05 h), which was significantly different (p-value < 0.05) to the estimated lag phases of LG160 and sCP034, which were 20 h and 25 h, respectively. The rest of the strains showed a period of lag phase between 9 h to 16 h when growing on this media. Despite sCP107 exhibiting a µ of 0.15 h^{-1}, this was only significantly different from the μ estimated for SCP047 (0.02 h^{-1}), but no significant differences were observed compared to the rest of the strains (µ values between 0.03 h^{-1} and 0.10 h^{-1}).

Remarkably, sCP041 and sCP047 did not show any advantage when grown in DM-glucose. In contrast, the engineered strains expressing an extra copy of the transporter *AGT1* (sCP101 and sCP107) displayed a greater ability to grow on DM-glucose. Even though these strains showed a more extended lag phase (10 h to 12 h), they exhibited a growth rate of 0.10 h^{-1} and 0.11 h^{-1} which was significantly different (p-value < 0.05) to the rest of the strains. Also, the maximum population size reached by sCP101 and sCP107 (OD_{600} 0.46 to 0.50) was significantly different (p-value < 0.01) to the *K* values estimated for the rest of the strains (OD_{600} 0.31 to 0.34).

4. Discussion

To examine the effects of sugar consumption by lager yeast that overexpress *IMA1* and *AGT1*, integration of an additional copy of each of the genes under the control of strong constitutive promotes into defined loci in the genome of *S. pastorianus* is desirable. However, to our knowledge, no genetic toolbox is available for this purpose. We therefore first implemented a platform for heterologous gene expression for *S. pastorianus* inspired by a genetic toolbox for *S. cerevisiae* originally developed by [54] and further developed into EasyClone [54,69–71]. In the platform for *S. cerevisiae*, the 16 sites, selected for high expression levels, are organized in clusters and located on three different chromosomes. Genes in the clusters are closely linked and tend to stay together during meiotic segregation. This feature allows genes of interest (GOI), that have been integrated in different clusters

in separate strains, to recombine during sexual crossing. If GOIs controlled by the same promoter and/or terminator are integrated in the same clusters, the cluster will contain direct repeats, which may lead to undesirable GOI loss due to direct repeat recombination. However, as the regions between integration sites contain an essential gene, these recombinant strains will not lose these specific regions.

Inspired by these studies, we decided to establish a versatile gene expression platform suitable for the two allodiploid LG160 and LG230 lager yeast. This platform considers five integration sites located as two clusters on two chromosomes, see Figure 1b, to facilitate gene integration. However, the first step required the sequencing of the model lager yeast LG160 and LG230. For each site, a matching integration vector was shaped to facilitate construction of gene-targeting cassettes including the desired GOI(s), see Figure 1c. Each gene-targeting cassette comes with targeting sequences matching the site of integration, two terminators, and a *URA3* gene for selection flaked by direct repeats. Hence, by using a single- or a bidirectional promoter, one or two ORFs can be expressed from each cassette. All DNA parts for vector construction can easily be assembled by USER^{TM} fusion [55] in a single cloning step, or by similar seamless DNA fusion technologies [72–74].

The genome sequencing of the parental strains served two purposes. First, it made the verification of endogenous copies of *IMA1* and *AGT1* in the parental strains possible. Nonetheless, in *Saccharomyces* species, the correct identification of the genes related to maltose and maltotriose assimilation is challenging due to their chromosomic location [5,75,76]. Second, the insertion of additional copies of *IMA1* and *AGT1* required the identification of identical integrations sites shared by both parental strains.

The estimated growth parameters of sCP034, expressing an extra copy of *IMA1*, which encodes the major isomaltase Ima1, and the parental strains were similar [77] reported that the overexpression of *IMA1* was enough to enhance the growth rate of *S. cerevisiae* when growing in a media with sucrose as a carbon source. While *IMA1* is required for isomaltose degradation, other studies have demonstrated that Ima1 also exhibits specificity for maltose, palatinose, α-methylglucoside, α-methylglucopyranoside, and sucrose [35,36]. Our results are consistent with the study reported by [35]. According to these authors, the growth of *S. cerevisiae* in a media containing isomaltose as a carbon source requires the presence of both genes, *AGT1* and *IMA1*.

Considering the biomass formation as an indirect measure of the consumption of the available carbon source in the media, it can be inferred that sCP041 and sCP047 possess an improved ability to utilize panose. Importantly, such a phenotype was absentee in the rest of the engineered strains, in which single copies of *AGT1* or *IMA1* were integrated (sCP034, sCP101, and sCP107), and in the parental strains. These results demonstrate the synergistic effect of *AGT1* and *IMA1* in improving the attenuation panose by *S. pastorianus*.

To this day, only a limited number of studies have described panose consumption by brewing yeast. For example, [19] reported *S. cerevisiae* ale yeast that utilized panose and isomaltose at a different level depending on the strain. These authors described how some strains utilized isomaltose efficiently, but panose was consumed only with low efficiency by a small number of strains [19]. More recently, [33] reported superior wort attenuation by a de novo created lager hybrid. The transcriptomic analysis of the new hybrid, which grew in industrial beer wort, revealed that four genes, *DAL5, IMA1, AGT1,* and *PTR2*, were overexpressed. Outstandingly, the lager strain reported by [33] was generated via a classical genetic approach by hybridizing an ale *S. cerevisiae* strain with a lager *S. pastorianus* strain. It has been recently proposed by [14] to classify this type of hybrids as lager yeast group III. This new classification includes hybrids generated by breeding ale and lager yeast, resulting in lager yeast carrying unique genome architectures. The genomic composition of lager yeast group I is roughly 33% *S. cerevisiae* type and 66% *S. eubayanus* type, the one of group II is 50% *S. cerevisiae* type the rest *S. eubayanus* type, and the genome composition of group III is 75% *S. cerevisiae* type and 25% *S. eubayanus* type [14].

Engineered lager yeast expressing *AGT1* and *IMA1* exhibited superior ability to grow on panose and isomaltose, whereas the engineered strains expressing these genes

individually did not. A plausible explanation of what we observed in this study could be related to the total number of copies of *AGT1* and *IMA1* present in the genomes of lager yeast. Probably two copies of *IMA1* and one copy of *AGT1* in the genomes of the parental strains are insufficient to assimilate panose or isomaltose [33]. An alternative explanation could be related to the transcriptional regulators of these genes. In *S. cerevisiae*, *MALR* genes encode for the regulator required to activate the expression of *MALT* and *MALS* [35,39,43,44]. Likewise, although the Himalayan *S. eubayanus* strain has several copies of *AGT1*, this yeast is unable to grow on maltose or maltotrioses. Yet, the expression of a functional copy of a *MALR*, from *S. cerevisiae*, enabled *S. eubayanus* to propagate on oligoglucosides [39]. In this study, the transcriptional activation by *MALR* is unnecessary as strong constitutive promoters control the expression of the genes.

In summary, this study presented the design and proof of concept of a gene integration toolbox suitable for lager yeast auxotrophic to uracil. Also, it was demonstrated that stable integration of a single copy of *AGT1* improved the maximum growth rate and the maximum population size of *S. pastorianus* when using glucose as a carbon source. Moreover, the integration of *IMA1* and *AGT1* improved the growth of lager yeast on panose and isomaltose, a phenotype not presented by the strains carrying single copies of the genes, which indicated a clear synergistic effect of *AGT1* and *IMA1* on the utilization of these oligosaccharides. Superior utilization of these sugars might impact industrial beer brewing processes by increasing the ethanol yield or modifying the organoleptic traits of the final product. Finally, strategies to generate industrial lager yeast capable of employing panose and isomaltose as carbon sources might include identifying strains overexpressing *AGT1* and *IMA1*.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7030107/s1, Figure S1: Representation of the location of the integration sites designed for the model lager yeast LG160 and LG230, Figure S2: Neighbor joining phylogenetic trees of several isomaltases, Table S1: Nucleotide sequences of lager yeast integration sites, Table S2: List of primers used in this study. Table S3: List of biobricks used in this study. Table S4: List of plasmids used in this study. Table S5: Nucleotide sequences of the synthetic isomaltase and maltose transporter genes, Table S6: Growth parameters, Table S7: Summary of the statistics of the genome assemblies.

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