RESEARCH

QTL mapping of a Brazilian bioethanol strain links the cell wall protein-encoding gene GAS1 to low pH tolerance in *S. cerevisiae*

Alessandro L. V. Coradini[1,2†], Fellipe da Silveira Bezerra de Mello[1†], Monique Furlan[1†], Carla Maneira[1], Marcelo F. Carazzolle[1], Gonçalo Amarante Guimaraes Pereira[1*] and Gleidson Silva Teixeira[1]

Abstract

Background: *Saccharomyces cerevisiae* is largely applied in many biotechnological processes, from traditional food and beverage industries to modern biofuel and biochemicals factories. During the fermentation process, yeast cells are usually challenged in different harsh conditions, which often impact productivity. Regarding bioethanol production, cell exposure to acidic environments is related to productivity loss on both first- and second-generation ethanol. In this scenario, indigenous strains traditionally used in fermentation stand out as a source of complex genetic architecture, mainly due to their highly robust background—including low pH tolerance.

Results: In this work, we pioneer the use of QTL mapping to uncover the genetic basis that confers to the industrial strain Pedra-2 (PE-2) acidic tolerance during growth at low pH. First, we developed a fluorescence-based high-throughput approach to collect a large number of haploid cells using flow cytometry. Then, we were able to apply a bulk segregant analysis to solve the genetic basis of low pH resistance in PE-2, which uncovered a region in chromosome X as the major QTL associated with the evaluated phenotype. A reciprocal hemizygosity analysis revealed the allele *GAS1*, encoding a β-1,3-glucanosyltransferase, as the casual variant in this region. The *GAS1* sequence alignment of distinct *S. cerevisiae* strains pointed out a non-synonymous mutation (A631G) prevalence in wild-type isolates, which is absent in laboratory strains. We further showcase that *GAS1* allele swap between PE-2 and a low pH-susceptible strain can improve cell viability on the latter of up to 12% after a sulfuric acid wash process.

Conclusion: This work revealed *GAS1* as one of the main causative genes associated with tolerance to growth at low pH in PE-2. We also showcase how *GAS1*[*PE-2*] can improve acid resistance of a susceptible strain, suggesting that these findings can be a powerful foundation for the development of more robust and acid-tolerant strains. Our results collectively show the importance of tailored industrial isolated strains in discovering the genetic architecture of relevant traits and its implications over productivity.

*Correspondence: goncalo@unicamp.br
†Alessandro L. V. Coradini, Fellipe da Silveira Bezerra de Mello and Monique Furlan contributed equally to this work
[1] Department of Genetics, Evolution, Microbiology, and Immunology, Institute of Biology, University of Campinas, Rua Monteiro Lobato 255, Campinas 13083-862, Brazil
Full list of author information is available at the end of the article

Background

Saccharomyces cerevisiae strains that are resistant to acidic environments are desirable in many relevant biotechnological processes, from probiotics, food and beverage industries [1–3], to bioethanol production [4, 5]. Usually, bioethanol is produced from a fermentation process driven by *S. cerevisiae* in which carbon sources from raw feedstock, such as corn, beet, wheat, and sugarcane are employed [6]. The alcohol

Coradini *et al. Biotechnology for Biofuels* (2021) 14:239

Page 2 of 16

production is based on the fermentation of available 6-carbon sugars from juice and/or starch (first-generation ethanol, E1G), or 5 and 6-carbon sugars present in lignocellulosic material and made available through hydrolysis (second-generation ethanol, E2G) [7]. In the latter, acidic fermentation environments arise as a consequence of the acid treatment of lignocellulosic material, which produces high quantities of acetic acid and other inhibitory by-products, such as 5-hydroxymethylfurfural and furfural [8]. The combination of low pH and inhibitory by-products perturb the intracellular pH homeostasis, inducing cell death and consequent loss of productivity [9, 10]. Therefore, an additional step of pH neutralization is often required before proceeding to the fermentation process increasing the operational costs [11].

In Brazil, the second-largest bioethanol producer in the world, distilleries commonly use a Melle-Boinot fermentation operation [12], in which high-density cell cultures are applied and the yeast recycled by centrifugation and acid washing to initiate a subsequent fermentation. In short, yeast cells are exposed to an acid treatment with dilute sulfuric acid (H_2SO_4) for up to 2 h before being reintroduced into a new vessel for a subsequent cycle of fermentation [13]. This unit operation aims to reduce bacterial contamination and prepare the cells for a new fermentation batch, reducing the need for yeast propagation, thus generating a more productive and less consuming process [14]. However, the severity of the process decreases the viability of the yeast population and consequently reduces productivity [15]. Also, this process may work as a cell bottleneck, limiting the variety of strains that can be used in the process [16].

Given the harsh conditions faced by yeasts in the Brazilian industrial fermentation process, indigenous strains have been praised for their robust background that allows higher ethanol productivity while facing burdening stress factors. In this scenario, *S. cerevisiae* strain Pedra-2 (PE-2) has been described as efficient industrial yeast, able to outperform native yeasts and dominate the fermentation process within a few cycles of fermentation and recycling [5, 17, 18]. The molecular analysis of JAY270 (PE-2 industrial isolate) shed light on its highly heterozygous genome architecture, which harbors structural polymorphisms between homologous chromosomes, especially in peripheral regions. The extreme heterozygosity and plasticity of the PE-2 genome and transcriptome have been hypothesized to contribute to its rapid adaptability to the industrial fermentative environment [19]. In fact, analysis of PE-2 performance during growth at harsh condition demonstrated its superior phenotype when exposed to low pH [17, 20], high temperature and oxidative stress conditions [19].

Responses to acid stress in *S. cerevisiae* have been thoroughly revised elsewhere [21]. Besides the effects on cell viability [22] and fermentation productivity [23], low pH can induce oxidative stress [24] and enhance ethanol toxicity [25].

Acid stress can be triggered by the presence of week organic acids (WOA) such as lactic, acetic and formic acid or by inorganic acids mainly sulphuric and hydrochloric. The presence of WOA at external pH below the weak acid pK_a value, facilitate the entrance of the undissociated form of the acid (RCOOH) on the cell by simple diffusion [1]. Once in the neutral cytosol, the chemical dissociation of the weak acid occurs leading to the release of protons (H^+) that strongly decrease the pH of cell cytoplasm [26]. Thus, the main cell response to this internal acidification involves increasing vacuolar H^+-ATPase (V-ATPase) activity [27–34]. Due to the high energetic cost of proton cell extrusion, additional mechanisms to prevent cytosolic acidification are necessary which include alteration of the molecular composition and physical properties of plasma membrane and cell wall [1]. In this scenario, genes related to cell wall remodeling and synthesis of cell wall major polysaccharides—chitin and β-glucan, and polymers of mannose—are transcriptionally responsive to weak acids [29, 35–39]. Stiffness of the cell wall caused by an increased content of cell wall β-glucans was recently associated to yeast resistance to acetic acid [40] demonstrating that cell wall remodeling may also play an important role on cell response to acid stress by WOA.

The capacity to maintain and remodel cell wall also appears as the main physiological response to yeast adaptation to inorganic acid exposure. When an inorganic acid is present in the environment, a high concentration of protons is generated. However, distinct from WOA, protons diffuse poorly across the plasma membrane and so the cytoplasm pH is not drastically reduced [41]. Thus, cell exposure to inorganic acids mainly affects cell wall structure and organization [4, 41, 42]. Previous studies on yeast transcriptional response to low pH demonstrated that adaptation of yeast involves mechanisms that include induction of cell wall integrity (CWI) genes and general stress response (GSR) pathway mainly due to the action of protein kinase C (PKC) [36, 42–45]. In particular, genes related to transport, protein anchoring and synthesis of the β-1,3-glucan chain on yeast cell wall are up-regulated after cell treatment with sulphuric acid [42, 44]. Together these results point to the possibility of the cell wall remodeling, in special changes in its polysaccharides content, as a general response of the cell to acidic environment. Therefore, changes in cell wall structure and content induced by exposure to inorganic acids

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 3 of 16

may also lead to an improved resistance to growth in an acidic environment in the presence of either inorganic or organic acids.

In this study, we performed a Quantitative Trait Loci (QTL) mapping approach to unravel the genetic architecture behind the high acid resistance phenotype of the PE-2 strain. We first hypothesized that currently exposure of PE-2 to repetitive cycles of acid treatment with dilute sulfuric acid (H_2SO_4) in Brazilian bioethanol mills conferred to PE-2 an increased tolerance to low-pH environments. This hypothesis was confirmed by the superior performance of PE-2 over other 41 Saccharomyces sp. strains during growth at low pH (2.5). Using a high-throughput approach to en masse phenotype of segregants at pH 2.1 followed by a bulk segregant analysis approach (BSA), allows the identification of 2 major *loci* on chromosome X and XIII. Furthermore, Reciprocal hemizygosity analysis (RHA) revealed the *GAS1* allele from PE-2 as the main causal variant associated to loci on Chromosome X. Sequence alignment of different *S. cerevisiae GAS1* alleles pointed out a persistent non-synonymous mutation within industrial and wild-type isolates, in contrast to laboratory strains. Finally, we showcased that low pH resistance phenotype can be recovered in susceptible strains with the insertion of PE-2 mutated *GAS1*. So far, this study presents itself as the first to explore the genetic basis of tolerance to growth at low pH in *S. cerevisiae* using a QTL mapping approach. Also, this work may help to better understand the molecular mechanisms underlying relevant industrial traits and, consequently,
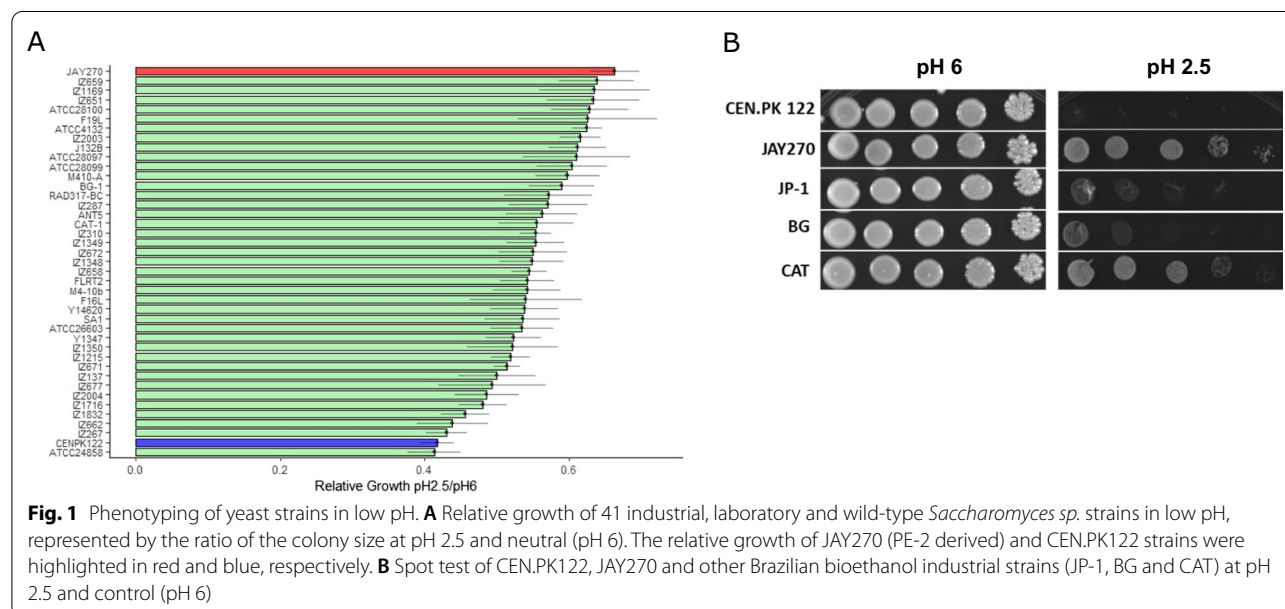
could foment the development of more robust strains for different applications.

Results

Screening of parent strains for genetic mapping

The growth performance of the PE-2 isolate, JAY270 (MATa/MATα), and other 40 *Saccharomyces sp.*—including laboratory, industrial and wild-type isolates—was assessed by measuring their relative colony size during growth on rich media at low (2.5) and neutral (6) pH (Fig. 1A). The JAY270 strain displayed the best growth performance in comparison to the other evaluated strains, presenting only 33% colony size reduction at low pH. On the other hand, the laboratory strain CEN.PK112 [46] ranked amongst the strains with the lowest performance. JAY270 also outperforms other typically used bioethanol strains (Fig. 1B). Therefore, considering tolerance to growth at low pH, JAY270 and CEN.PK112 were selected as the superior and inferior strain, respectively.

JAY270 segregant haploids were collected using fluorescence-activated cell sorting (FACS) by flow cytometry through a distinct mating-type approach. For this, we constructed vector pMF_002, comprising the reporter gene enhanced green fluorescent protein (EGFP) fused to the MATa-specific STE2 (STErile 2) promoter, and cyan orange fluorescent protein (CyOFP1), to MATα-specific STE3 (STErile 3) promoter (see "Methods" for details). When using only a fluorescein isothiocyanate (FITC) filter, EGFP and CyOFP1 are excited in distinct wavelengths of 515/545 (green) and 655/695 (orange), respectively. Thus, when expressing pMF_002, MATa cells emit green fluorescence and MATα cells orange

Fig. 1 Phenotyping of yeast strains in low pH. **A** Relative growth of 41 industrial, laboratory and wild-type *Saccharomyces sp.* strains in low pH, represented by the ratio of the colony size at pH 2.5 and neutral (pH 6). The relative growth of JAY270 (PE-2 derived) and CEN.PK122 strains were highlighted in red and blue, respectively. **B** Spot test of CEN.PK122, JAY270 and other Brazilian bioethanol industrial strains (JP-1, BG and CAT) at pH 2.5 and control (pH 6)

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 4 of 16

fluorescence, while diploid cells (MATa/MATα) and tetrads do not display any fluorescence. This technique allowed the separation and distinct collection of 1084 stable JAY270 haploid cells (Fig. 2). A similar strategy has been previously published by Treusch et al. [47], and here adapted to be suitable for more commonly available excitation wavelengths.

Before proceeding to the phenotyping of the isolated segregants, a minimum inhibitory concentration (MIC) was established for acid tolerance—i.e. the minimum pH value capable of totally inhibiting the growth of at least one haploid cell. Therefore, the colony size of 48 randomly selected JAY270 segregants was evaluated in decreasing values of pH (Additional file 1: Fig. S1), and a pH of 2.1 was selected as the MIC. Next, the panel of 1,084 JAY270 collected haploids was tested for their growth at pH 2.1 to evaluate their phenotypic response.
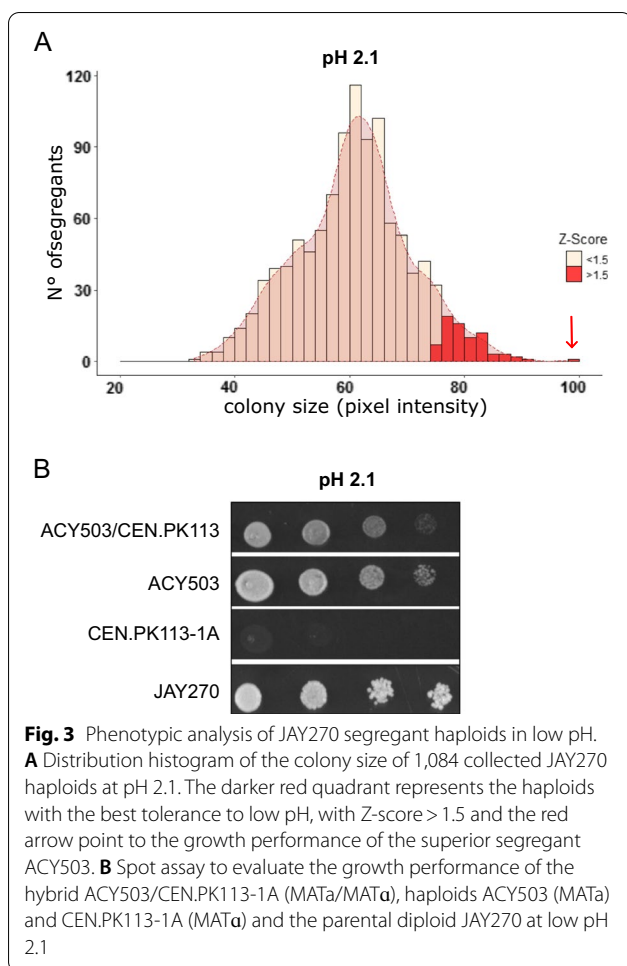
The normal distribution of the segregants' colony size in the evaluated condition reveals the quantitative characteristic of the low pH tolerance phenotype in the JAY270 strain (Fig. 3A). In this population, haploid ACY_503 (MATa) presented the largest colony size at pH 2.1, therefore was selected as the superior parental strain. Strain CEN.PK113-1A (MATα), CEN.PK122 segregant that presented reduced growth at low pH, was chosen as the inferior parental strain. The crossing between the selected strains resulted in the diploid ACY_503/CEN.PK113-1A. The hybrid diploid and both parental strains were submitted to a colony spot assay to confirm their phenotypes in an acid medium (Fig. 3B). As expected, the resistance and susceptibility of parental haploid strains ACY_503 and CEN.PK 113-1A, respectively, was confirmed. In addition, it is important to notice that the resulting hybrid ACY_503/CEN.PK113-1A presented low pH resistance similar to the superior parental ACY_503, indicating that the genetic architecture that underlies the acid tolerance in JAY270 should be dominant.
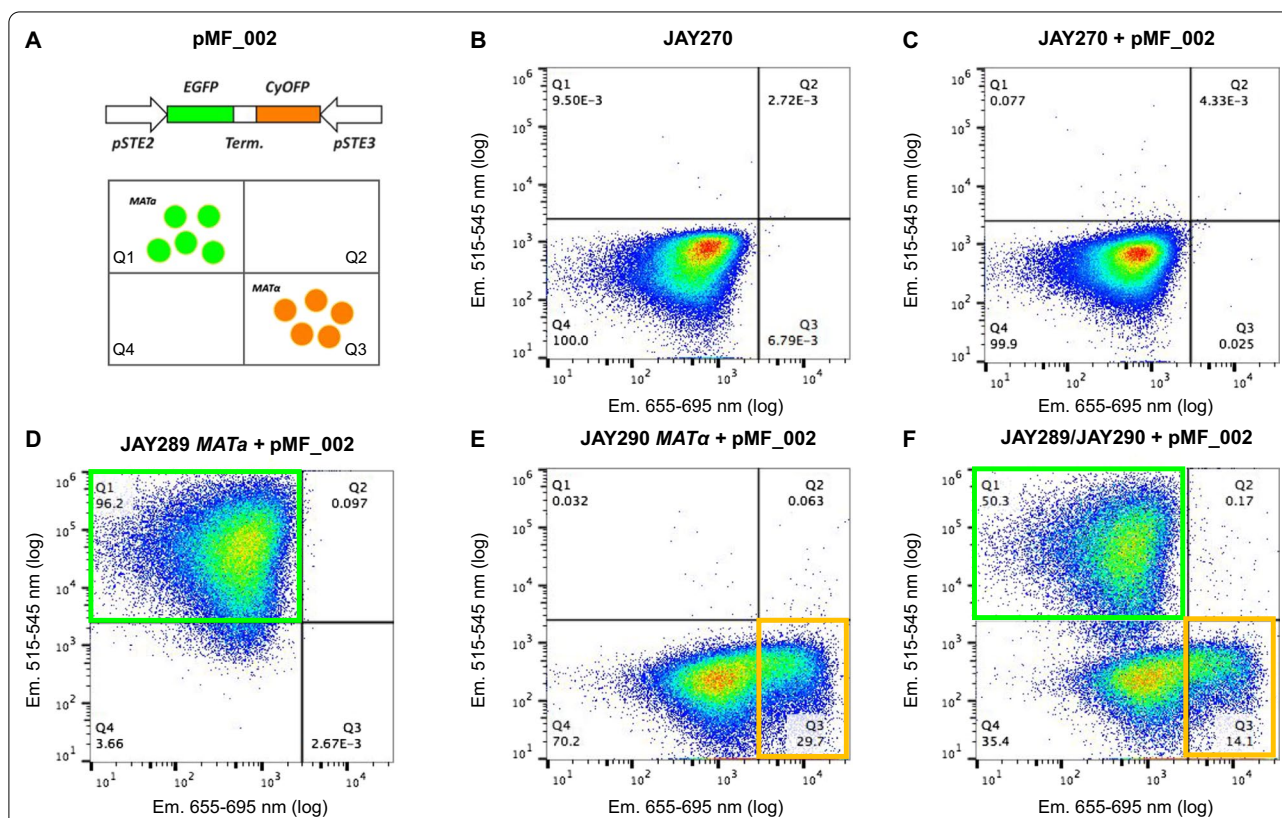
Fig. 2 Patterns of fluorescence emission in strains transformed with pMF_002 vector and analyzed by flow cytometric. **A** Schematic representation of the pMF_002 vector and simplification of expected results. **B** Fluorescence pattern of the diploid strain JAY270 without pMF_002 vector. **C** Fluorescence pattern of the diploid strain JAY270 containing the vector pMF_002. **D** Fluorescence pattern of the haploid strain JAY289 (MATa) transformed with vector pMF_002. **E** Fluorescence pattern of the haploid JAY290 (MATα) transformed with pMF_002. **F** Fermentation pattern of a heterogeneous population comprising both haploids JAY289 (MATa) and JAY290 (MATα) transformed with vector pMF_002. Specific MATa cells emitting green fluorescence are expected to form a population on quadrant 1 (Q1) while specific MATα cells emitting orange fluorescence are expected to fall on quadrant 3 (Q3)

Fig. 3 Phenotypic analysis of JAY270 segregant haploids in low pH. **A** Distribution histogram of the colony size of 1,084 collected JAY270 haploids at pH 2.1. The darker red quadrant represents the haploids with the best tolerance to low pH, with Z-score > 1.5 and the red arrow point to the growth performance of the superior segregant ACY503. **B** Spot assay to evaluate the growth performance of the hybrid ACY503/CEN.PK113-1A (MATa/MATα), haploids ACY503 (MATa) and CEN.PK113-1A (MATα) and the parental diploid JAY270 at low pH 2.1

Fig. 4 En masse selection strategy to obtain a pool of low-pH-tolerant segregants from the ACY503/CEN.PK113-1A cross. **A** Schematic view of an en masse collection of a pool of ACY503/CEN.PK113-1A acid-tolerant segregants. For the QTL mapping, initially a highly low-pH-resistant JAY270 segregant was selected (ACY503) and further crossed with a haploid of the opposite phenotype. The hybrid transformed with pMF_002 was sporulated and segregants were further collected using a cell sorter coupled with a flow cytometer in growing challenges of pH resistance. **B** Image of YPD plates with decreasing values of pH (4, 3, 2.5 and 2.1) where 500.000 ACY503/CEN.PK113-1A segregants were collected in flow cytometry. 79 segregants obtained at pH 2.1 were considered a *"high resistance pool"* and used for QTL mapping
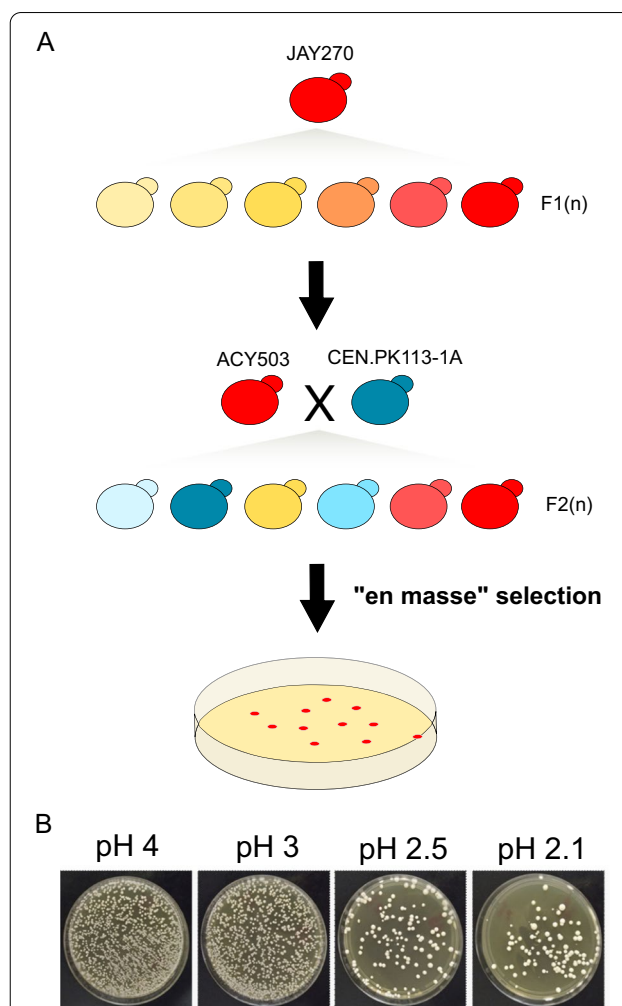
Selection of the two pools of segregants with extreme phenotypes

The use of the plasmid pMF_002 allowed the application of the bulk segregant analysis (BSA) approach to map potential QTL related to tolerance to growth at low pH on PE-2. Therefore, ACY_503/CEN.PK113-1A was transformed with the vector pMF_002, sporulated and its segregants were collected using a cell sorter coupled to a flow cytometer. In order to select segregants in the positive extreme of low pH tolerance phenotypic distribution, the isolated F_2 population was collected on YPD plates containing decreasing values of pH: 4, 3, 2.5, and 2.1 (Fig. 4). In this scenario, after 96 h of incubation, 79 segregants—*petite* colonies were excluded from this examination—were obtained and labeled as the *"high resistance pool"* (Additional file 2: Fig. S2A).

One disadvantage of BSA is that it does not allow for the selection of a pool of segregants with inferior phenotype (i.e. haploids susceptible to low pH), since en masse selection is provided by increasing the restrictive growth condition. Thus, we randomly selected 500 haploids that were further phenotyped using a colony spot assay, resulting in 79 segregants with reduced growth at pH 2.1, that were labeled as the *"low resistance pool"* (Additional file 2: Fig. S2B).

Identification of QTL related to growth tolerance at low pH of the JAY270 strain

Genomic DNA of four samples: (i) PE-2-derived superior haploid ACY_503; (ii) laboratory susceptible haploid

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 6 of 16

CEN.PK113-1A; (iii) 79 segregants from the *high resistance pool*; and (iv) 79 segregants from the *low resistance pool*—were subjected to whole-genome sequencing analysis using the Illumina HiSeq 4000 platform that generated millions of 2×100 paired-end reads and genome coverages of 213, 170, 267, and 210x, respectively. The reads from the two parental strains and the two pools were first aligned to the CEN.PK113-7D reference genome sequence [48] to identify single nucleotide polymorphisms (SNPs). A total of 47,659 highly credible SNPs between high and low resistance pools were selected for QTL analysis.

The G′ values for each SNP were calculated using an 80-kb sliding window, and the p-value graph was plotted to identify the candidate peaks (Fig. 5). Peak regions of $- \log_{10}$(p-value) above the threshold of 2.3 were defined as candidate QTL regions comprising alleles responsible for the evaluated phenotype. The mapping shows two major peaks: at chromosome X and XIII with $- \log_{10}$ (p-value) of 6.12 and 4.7, respectively. Because chromosome XIII presented the highest value of $- \log_{10}$(p-value), therefore representing a region enriched with SNPs more statistically relevant in the *high resistance pool*, it was selected as the major QTL and used in further analysis.
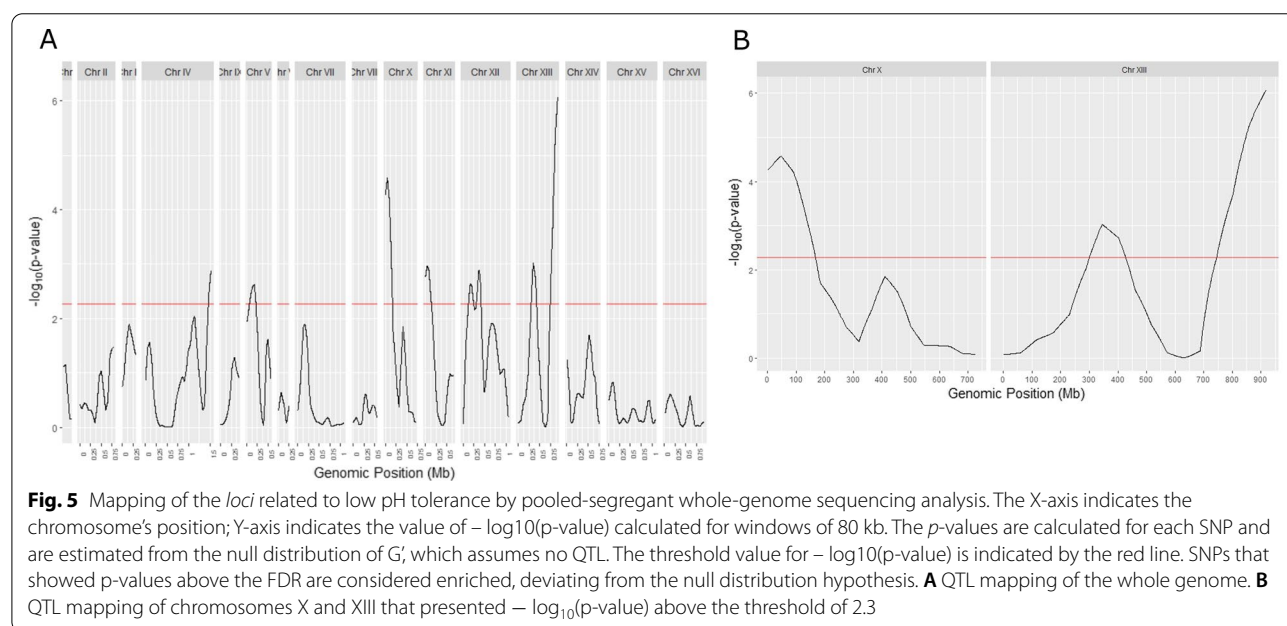
The mutations present within a region of 50 kb surrounding the highest peak position on chromosome XIII (897,054 bp) were analyzed and annotated. Initially, all SNPs located in non-coding regions and that confer synonymous mutations were excluded from the list of potential candidates. Further, we used the genome sequence of 14 *S. cerevisiae* strains, available at Saccharomyces

Genome Database (SGD) [49], to infer the non-synonymous coding SNP frequency in this population (Table 1). Since extreme low pH tolerance is not a common trait in yeast a low frequency of the candidate SNP was expected. Mutations with frequencies lower than 30% were, therefore, classified as potential candidates. Finally, we also considered the attributed function for each gene in which the filtered SNP is located to narrow down the number of potential causative alleles.

Using this approach, we were able to identify 4 potential candidate genes on chromosome XIII related to low pH resistance: *GAS1, ELP6, GLC8,* and *FET4*. A description of the function of each gene and the non-synonymous uncommon mutations present in ACY_503 can be found in Table 2.

Validation of the causative genes within chromosome XIII QTL

The validation of the candidate alleles was performed through RHA. Four pairs of hemizygous ACY_503/CEN.PK113-1A strains were generated, in which each pair retained a single copy of the superior ACY_503 or inferior CEN.PK113-1A parental alleles—*GAS1*, ELP6, *GLC8* or FET4 (Fig. 6A). The hemizygous strains for each candidate allele were tested for growth at pH 2.1 and the phenotypic response assessed through colony spot assays. The results showed that phenotypic variance is present on *GAS1* hemizygotes, and that the strain expressing the *GAS1*[ACY_503] allele presented superior growth compared to the one that harbors *GAS1*[CEN.PK113-1A]. On the other hand, the response of the other hemizygous diploid

Fig. 5 Mapping of the *loci* related to low pH tolerance by pooled-segregant whole-genome sequencing analysis. The X-axis indicates the chromosome's position; Y-axis indicates the value of – log10(p-value) calculated for windows of 80 kb. The *p*-values are calculated for each SNP and are estimated from the null distribution of G′, which assumes no QTL. The threshold value for – log10(p-value) is indicated by the red line. SNPs that showed p-values above the FDR are considered enriched, deviating from the null distribution hypothesis. **A** QTL mapping of the whole genome. **B** QTL mapping of chromosomes X and XIII that presented $- \log_{10}$(p-value) above the threshold of 2.3

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 7 of 16

Table 1 Non-synonymous mutations present in genes within a 50-kb window in chromosome XIII peak (897,054 bp) of low-pH phenotype QTL mapping

	YME2					UBP15			GAS1	NIP1
	255	817	1270	1408	2037	916	2410	2589	631	1388
S288C	A	G	A	G	G	C	A	A	A	T
CEN.PK	G	T	T	A	T	T	T	T	A	T
ACY503	A	G	A	G	G	C	A	A	G	A
X2180-1A	A	G	A	G	G	C	A	A	A	T
SEY6210	A	G	A	G	G	C	A	A	A	T
W303	A	G	A	G	G	C	A	A	A	A
JK9-3d	A	G	A	G	G	C	A	A	A	T
FL100	A	G	A	G	G	T	T	T	A	A
D273-10B	A	G	A	G	G	C	A	A	A	T
Sigma1278b	A	G	A	G	G	C	A	A	A	T
RM11-1a	G	T	T	A	T	C	A	A	G	A
SK1	G	T	T	A	T	C	T	A	G	A
Y55	G	T	T	A	T	C	A	A	G	A
BY4741	A	G	A	G	G	C	A	A	A	T
BY4742	A	G	A	G	G	C	A	A	A	T

	YMR310C		GLC8	ELP6	TGL3	DIA1		FET4	
	1776	442	605	685	4	1495	707	950	1656
S288C	T	T	C	C	G	T	T	C	T
CEN.PK	T	T	C	C	G	T	T	C	T
ACY503	C	C	G	A	A	C	C	T	C
X2180-1A	T	T	C	C	G	T	T	C	T
SEY6210	C	T	C	C	G	T	T	C	T
W303	T	C	G	A	A	C	C	C	T
JK9-3d	C	T	C	C	G	T	T	C	T
FL100	T	C	G	A	A	C	C	C	T
D273-10B	T	T	C	C	G	T	T	C	T
Sigma1278b	T	T	C	C	G	T	T	C	T
RM11-1a	T	C	C	C	G	C	C	C	T
SK1	C	C	G	A	A	C	C	C	T
Y55	T	C	C	C	A	C	C	C	T
BY4741	T	T	C	C	G	T	T	C	T
BY4742	T	T	C	C	G	T	T	C	T

strains for the remaining 3 genes not changed on the evaluated condition, compared to the wild-type hybrid.

Next, we sought to identify if swapping *GAS1* allele between susceptible and tolerant parental would induce different responses in their acid tolerance phenotype (Fig. 6B). Interchanging the *GAS1* allele between both strains significantly improves CEN.PK113-1A tolerance to low pH, while it reduces ACY_503's, corroborating with the results obtained by RHA and strongly suggesting that the *GAS1* allele contributes to low pH tolerance in segregant ACY_503, and consequently in PE-2 strains.

Compared to the CEN.PK113-1A allele, the *GAS1* allele of ACY_503 contains one non-synonymous point mutation, within its coding sequence (position 887,003 bp on chromosome XIII). This mutation accounts for a nucleotide exchange at position 631 from adenine to guanine and results in a threonine to alanine substitution. The *GAS1* allele from ACY_503 also harbors a synonymous mutation at position 1314 bp (i.e. a cytosine replacing thymine).
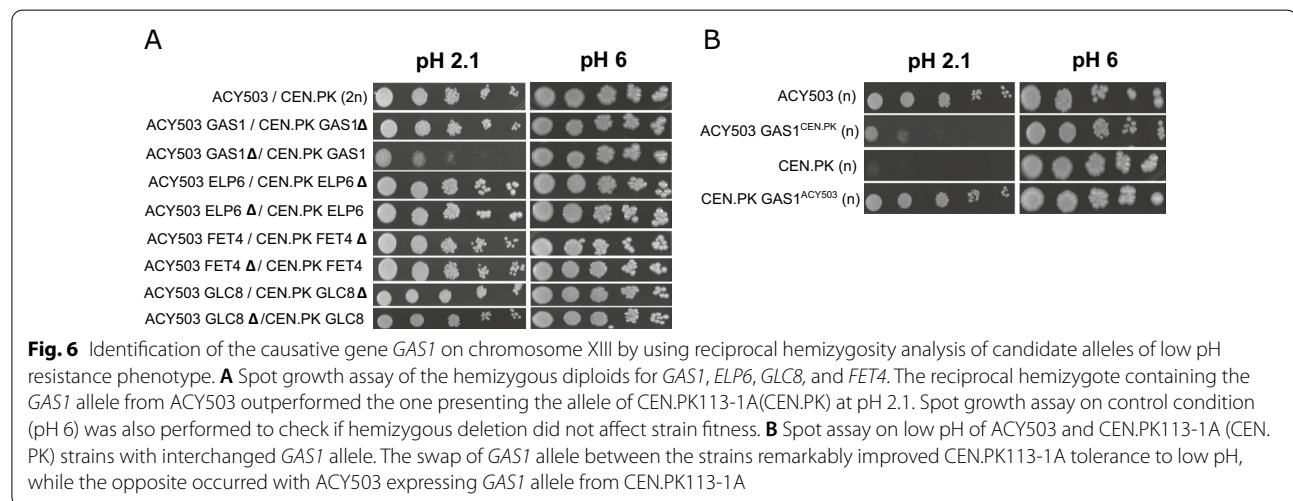
Next, we compared GAS1 sequence from ACY_503 and other 1053 *Saccharomyces cerevisiae* strains containing

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 8 of 16

Table 2 Possible causative alleles related to low-pH-resistance phenotype at chromosome XIII with uncommon non-synonymous coding mutations

Gene	Function[a]	Mutations in ACY503[b]
GAS1	Beta-1,3-glucanosyltransferase; required for cell wall assembly and also has a role in transcriptional silencing; genetic interactions with histone H3 lysine acetyltransferases GCN5 and SAS3 indicate previously unsuspected functions for Gas1 in DNA damage response and cell cycle regulation	A631G
ELP6	Elp6p is part of the six-subunit elongator complex, which is a major histone acetyltransferase component of the RNA polymerase II holoenzyme responsible for transcriptional elongation	C685A
GLC8	Regulatory subunit of protein phosphatase 1 (Glc7p); involved in glycogen metabolism and chromosome segregation; proposed to regulate Glc7p activity via conformational alteration; protein abundance increases in response to DNA replication stress	C605G
FET4	Although originally identified as a low-affinity iron(II) permease, Fet4p has since been shown to import several other transition metal ions, including copper and zinc	C950T; T1656C

[a] Available at https://www.yeastgenome.org/

[b] Non-synonymous uncommon mutations

Fig. 6 Identification of the causative gene *GAS1* on chromosome XIII by using reciprocal hemizygosity analysis of candidate alleles of low pH resistance phenotype. **A** Spot growth assay of the hemizygous diploids for *GAS1*, *ELP6*, *GLC8*, and *FET4*. The reciprocal hemizygote containing the *GAS1* allele from ACY503 outperformed the one presenting the allele of CEN.PK113-1A(CEN.PK) at pH 2.1. Spot growth assay on control condition (pH 6) was also performed to check if hemizygous deletion did not affect strain fitness. **B** Spot assay on low pH of ACY503 and CEN.PK113-1A (CEN. PK) strains with interchanged *GAS1* allele. The swap of *GAS1* allele between the strains remarkably improved CEN.PK113-1A tolerance to low pH, while the opposite occurred with ACY503 expressing *GAS1* allele from CEN.PK113-1A

Table 3 Evaluation of GAS1 SNPs frequency in 1053 distinct *Saccharomyces cerevisiae* strains

SNP location in GAS1	Nucleotide	Total *Saccharomyces cerevisiae* population		Brazilian bioethanol strain population	
		Number of strains	SNP frequency (%)	Number of strains	SNP frequency (%)
631	A (reference)	32	3.0	0	0
	G	1019	96.8	39	100
	R (A or G)	2	0.2	0	0
1314	T (reference)	54	92.0	7	17.9
	C	969	5.1	30	76.9
	Y (C or T)	30	2.8	2	5.1

laboratorial and wild isolate whose genome sequencing is available at Saccharomyces genome database (SGD) [49] and Peter et al. [50]. The frequency of non-synonymous mutation found on ACY_503 is high (96.8%) in the total *S. cerevisiae* analyzed population (Table 3). Interestingly, this mutation seems common throughout the majority of wild-type isolated strains, including all the Brazilian bioethanol isolates, while it is not found on laboratory ones (Additional file 3: Table S1). The same analysis was carried out for the synonymous mutation at nucleotide 1314 and distinct from the non-synonymous one it presents a low frequency (5.1%) on total analyzed population. However, this frequency increases to 76.9% when the analyzed population includes only isolates from Brazilian Bioethanol process (Table 3).

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 9 of 16

Analysis of the CEN.PK113-1A *GAS1*[ACY_503] mutant tolerance to acid-wash treatment with H_2SO_4 solution.

As previously stated, the E1G production process in Brazilian mills has the peculiarity of using the Melle-Boinot operation, characterized by fed-batch fermentation cycles with high-density cell recycle. Yeast cells are recycled up to 3 times per day and, between each recycling step, face an acid-wash treatment with sulfuric acid (pH ~ 1.5) that aims to reduce bacterial contamination and flocculation [16, 51]. Thus, after linking the *GAS1* allele to PE-2 tolerance to growth at low pH, we sought to explore if it is possible to increase tolerance to an acid-wash treatment in a naturally susceptible strain after allele swap (i.e. by constructing a strain expressing *GAS1*[ACY503] allele).

For this purpose, wild-type CEN.PK113-1A strain and its mutant CEN.PK113-1A *GAS1*[ACY_503] were submitted to an acid treatment in which cells were exposed to a diluted H_2SO_4 solution with pH 1.5 for up to 3 h. Viability was assessed every hour, revealing that the CEN.PK113-1A strain containing the mutated *GAS1* allele presents superior cell viability when compared to the wild type. After 3 h of exposure to H_2SO_4 solution, CEN.PK113-1A cell containing GAS1 allele maintaining 12% more viable cells when compared to wild type CEN.PK113-1A (Fig. 7).

Discussion

QTL mapping approaches have been extensively used to unravel the genetic basis of complex traits in a wide range of organisms [52]. In the yeast *S. cerevisiae*, QTL mapping approaches have facilitated the association between genetic variants and industrially relevant traits [53]. In this scenario, wild-type isolated strains present a higher degree of genetic and natural selection-driven diversity

compared with domesticated laboratory ones, which facilitate their use to resolve the genetic basis of so-desired relevant traits. In this study, a bioethanol industrial strain—PE-2, isolated from Brazilian mills—was used to unravel potential genetic variants associated with tolerance to growth at low pH in *S. cerevisiae*.

An initial evaluation of growth performance of JAY270 (PE-2 derivate) and other 40 *S. cerevisiae* strains cultivated at a low pH condition confirmed the superior phenotype of PE-2, especially when compared to the laboratory strain CEN.PK122. In fact, PE-2 persistence on the ethanol production process in Brazilian mills has already been associated with its resistance to the acid-wash cell recycle step typically performed on Brazilian E1G production process [5, 20]. On the other hand, common laboratory strains such as CEN.PK122 are typically cultivated under standard conditions that include slightly acidic pH and consequently do not undergo natural selection for this specific condition. Our initial result corroborates with the idea that the harsh conditions faced by yeast strains during bioethanol production produce tailored strains that can easily outcompete non-adapted ones. Thus, industrial isolated strains from the Brazilian bioethanol process, such as PE-2, SA-1, CAT-1, BG-1 can be a good source of genetic variability to explore the genetic basis of industrial relevant traits.

In order to investigate the genetic architecture of PE-2 acid tolerance, we first developed a high-throughput fluorescence-based approach to isolate a large number of yeast segregants. In comparison to other BSA approaches, such as X-QTL [54], our method allows the rapid generation of large mapping populations without extensive strain engineering. Despite the similarity to the fluorescence-based approach described by Treusch et al. [47], our method has the advantage of decreasing the number of wavelength gates necessary for segregant isolation. It makes use of the eGFP and CyOFP1 fluorescent proteins, which are excited at wavelengths of 515/545 (green) and 655/695 (orange), respectively, via a single FITC filter. This is the first report of the use of CyOFP1 in a fluorescent-based approach for yeast cell separation.

The developed fluorescence-based approach was successfully applied to isolate a large pool of segregants for BSA-based QTL mapping. BSA is an efficient approach for detection of major QTLs associated with complex traits in yeast [55–63]. This approach relies on phenotyping a progeny from a cross and genotyping two subsets of these offspring presenting opposite phenotypes [64]. The developed fluorescence-based approach was successfully applied to isolate a large pool of segregants (500.000) from a cross between the ACY_503 (PE-2 derivative) and CEN.PK113-1A strains. The segregants were collected in crescent challenging conditions of low pH, which allowed
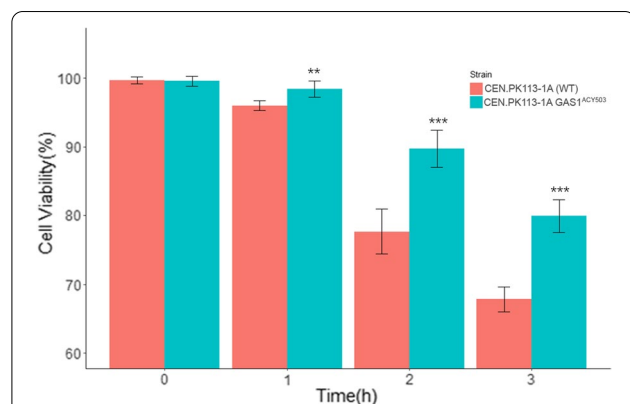
Fig. 7 Cell viability after acid-wash assay. Cell viability of CEN.PK113-1A and its mutant containing the *GAS1* allele from the tolerant strain ACY503 was assessed each hour during 3 h of treatment with H_2SO_4 solution

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 10 of 16

the selection of a pool of 79 superior and inferior haploids—representing less than 0.01% of the total analyzed population.

By analyzing differential SNPs presence in both pools (ΔSNP-index), we were able to identify a major QTL located at the end of chromosome XIII. Although the QTL region encompasses a genomic window of approximately 150 kb containing several genes, we focused our analysis on the 50 kb window surrounding the detected QTL peak. We also applied a protein function analysis as well as non-synonymous mutation information in these genes, narrowing down the number of potential candidates to only 4. Through generation of engineered reciprocal hemizygotes, we were able to identify *GAS1* as the causative allele on the major QTL at chromosome XIII. Furthermore, by interchanging the *GAS1* alleles between ACY_503 and CEN.PK113-1A, we remarkably increased CEN.PK113-1A tolerance to low pH condition. Both results indicate *GAS1* as the causal variant on QTL at chromosome XIII responsible for low pH tolerance on PE-2 strain.

GAS1 encodes a cell wall-bound 1,3-beta-glucanosyltransferase involved in the formation and maintenance of 1,3-beta-glucan, which is the major polysaccharide of the cell wall (see review on [65]). The Gas1p is a GPI-anchored glycoprotein of 125–130 kDa localized at the plasma membrane and is a member of the GH72 family of β-1,3-glucanosyltransferases that also include the *Candida albicans* pH-responsive proteins—CaPhr1p and CaPhr2p [66]. The enzyme is characterized by an N-terminal catalytic domain of about 310 residues (D23–P332), known as the β-(1,3)-glucan transferase domain (GluTD), a cysteine-rich region containing a motif of eight cysteines (C370–C462) and a serine-rich region in which 28 serines are clustered in a region between residues S485 and S525 [67].

A comparison between ACY_503 and CEN.PK113-1A alleles showed the presence of two distinct mutations at nucleotides positions 631 and 1314, non-synonymous and synonymous, respectively. Thus, the non-synonymous mutation at A211 amino acid residue is located at N-terminal catalytic domain and more precisely on α-helices domain between the two activity glutamates residues E161 and E262 necessary for *GAS1* activity as b-(1,3)-glucanase and b-(1,3)-glucanosyltransferase [68].

A more comprehensive analysis of the presence of both SNPs in other *S. cerevisiae* strains revealed that the non-synonymous mutation is common on wild strains isolated from distinct sources—e.g. wineries, bioethanol industries and oak trees. On the other hand, this mutation is absent in laboratory domesticated strains such as S288C, CEN.PK and W303. Yeasts are known to be organisms with the capacity to survive and ferment in a more acidic environment—pH 4–5 [41, 69]. Acidification of the extracellular environment can be a consequence of natural processes occurring during fermentation, presence of competing microorganisms producing organic acids or as a consequence of human interference during a biotechnological process [4]. However, acidic environments are rare in controlled laboratory conditions, where yeast growth is typically carried out in standard conditions that include neutral and controlled pH. The lack of selective pressure in laboratory growth conditions may have contributed to the loss of beneficial genetic variants associated with low pH resistance on laboratory strains.

Regarding the synonymous mutation at nucleotide 1314, it appears enriched in the Brazilian bioethanol strain population when compared to the total analyzed population (Table 3). This result may point to a positive selection of this mutation on the strains used in Brazilian bioethanol process. Although synonymous mutations are not expected to cause phenotypic changes, there is emerging evidence that it may affect cell fitness by altering translational efficiency, mRNA stability and also perturbing co-translational protein folding mechanisms [70–72].

The Gas1p role on yeast low pH resistance may be related to activation of cell wall integrity (CWI) pathway. The CWI pathway is responsible for maintenance and function of the yeast cell wall and its mechanism is controlled by the regulatory cascade led by protein kinase [73]. In summary, the stress sensor Mid2 mediates a response to acidic conditions that leads to activation of the Rlm1 transcription factor through phosphorylation of MAP kinase Slt2p/Mpk1p [74, 75]. *Gas1Δ* mutant strains show hypersensitivity to low pH and present higher levels of dually phosphorylated Slt2, which may help explain the connection between Gas1p and CWI pathway. Some studies have also demonstrated the existence of a synthetic interaction between Gas1p, Slt2p and Rlm1 [76]. Collectively these results point that maintenance of cell wall structure is an important response to low pH stress. Corroborating with this idea, transcriptomic analysis of yeast cells growing under low pH showed that GAS1 and other genes related to cell wall biogenesis appear up-regulated as a response to the damages caused by strong inorganic acids such as sulphuric acid [42], a stress response that may be triggered by CWI pathway activation.

Recently, Ribeiro et al. [40] demonstrated that Gas1p also play a role in yeast cell wall response to stress caused by organic acid such as acetic acid. The presence of 60 mM of acetic acid (pH 4.0) in the medium up-regulates the transcription of β-1,3-glucanosyltransferase encoded by gene *GAS1* that leads to an increased content of cell wall β-glucans. This correlation between the

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 11 of 16

increased levels of *GAS1*'s transcripts and the content of glucan in the cell wall suggests that at least partially, the cell wall remodeling under acetic acid presence is due to the action of β-1,3-glucanosyltransferase encoded by *GAS1*. This remodeling is essential for preventing acetate (dissociated form of acetic acid due to the low pH) entry through passive diffusion into the cell.

The *GAS1* was also identified as responsible for low pH resistance of the multiple-stress-tolerant yeast *Issatchenkia orientalis* (*Pichia kudriavzevii*) [68]. Matsushika et al. have screened on *S. cerevisiae* a genomic DNA library of *I. orientalis* identifying *loGAS1* as the allele responsible for low pH resistance and also demonstrating that expression of lo*GAS1* in *S. cerevisiae* (S288C) improved its ethanol fermentation ability at pH=2. In a complementary study, the same group demonstrated that *S. cerevisiae GAS1* (*ScGAS1*) expression is pH-dependent and increases in low pH conditions [77]. Also, overexpression of *ScGAS1* improved growth and ethanol production under acid stress conditions, although the stress tolerance was inferior to that of the *IoGAS1*-overexpressing strain. The DNA sequences of both genes—*loGAS1* and *ScGAS1*, possess approximately 60% of similarity [68]. Interestingly, by analyzing and comparing the amino acids profile from loGas1p with the Gas1p produced by the ACY_503 and CEN.PK113-1A alleles, we found that the non-synonymous mutation present in the ACY_503 allele result in the same amino acid (alanine) as at that position in the *loGAS1* gene.

Finally, to build evidence that $GAS1^{ACY\_503}$ may also play a role to PE-2 tolerance to acid-wash treatment of the cell recycle process on Brazilian bioethanol mills, we analyzed the cell survival rate of the susceptible strain CEN.PK113-1A and its mutant CEN.PK113-1A $GAS1^{ACY\_503}$ when submitted to a H_2SO_4 solution. The

result showed that the strain harboring mutant *GAS1* allele preserves up to 12% more viable cells after 2 and 3 h of acid treatment. This result might be indicative of PE-2 strain tolerance to the acid-wash treatment and its prevalence on fermentation vessels, as described by Basso et al. [16]. Furthermore, this result also opens the possibility of using genetic engineering to develop more robust strains for ethanol production (E1G and E2G) and also other biotechnological processes where cells experienced loss of cell viability or productivity due to the acidic environment.

Conclusion

In this study, we explored the industrial isolated strain PE-2 resistance to growth at low pH. By using a high-throughput approach, we were able to isolate and collect thousands of segregants and apply a BSA to map the QTL related to this phenotype. Following, a RHA approach allowed us to uncover the allele *GAS1* as one of the causal variant related to low pH tolerance by PE-2. Further, we used reverse genetic engineering to improve tolerance to acidic pH of the strain CEN.PK113-1A, demonstrating that $GAS1^{ACY\_503}$ is able to confer this phenotype. This study is, up to date, the first study that used the QTL approach to solve the genetic basis of tolerance to low pH in yeast. The knowledge provided here may help develop more robust strains for ethanol production and also other yeast-based industrial processes.

Methods

Strains and plasmids

A total of 41 *Saccharomyces sp.* strains (Additional file 4: Table S2) were used for the screening of low-pH tolerance phenotype in yeast. Diploid industrial *S. cerevisiae* PE-2 [19] was used as the acid-resistant reference strain,

Table 4 Main *Saccharomyces cerevisiae* strains used in this work

Strain	Description	Source
PE-2	Brazilian ethanol mill indigenous diploid strain	Basso et al. [5]
CEN.PK113-1A (CEN.PK)	*MATa* (prototrophic)	Euroscarf
ACY503	*MATa,* haploid segregant from PE-2	This study
ACY503/CEN.PK113-1A	Hybrid diploid strain from the crossing of ACY503 and CEN.PK113-1A	This study
ACY503 *gas1*/CEN.PK *gas1Δ*	ACY503 crossed with CEN.PK113-1A *gas1Δ*	This study
ACY503 *Δgas1*/CEN.PK *gas1*	ACY503 *gas1Δ* crossed with CEN.PK113-1A	This study
ACY503 *elp6*/CEN.PK *elp6Δ*	ACY503 crossed with CEN.PK113-1A *elp6Δ*	This study
ACY503 *Δelp6*/CEN.PK *elp6*	ACY503 *elp6Δ* crossed with CEN.PK113-1A	This study
ACY503 *fet4*/CEN.PK *fet4Δ*	ACY503 crossed with CEN.PK113-1A *fet4Δ*	This study
ACY503 *fet4Δ*/CEN.PK *fet41*	ACY503 *fet4Δ* crossed with CEN.PK113-1A	This study
ACY503 *glc8*/CEN.PK *glc8Δ*	ACY503 crossed with CEN.PK113-1A *glc8Δ*	This study
ACY503 *glc8Δ*/CEN.PK *glc8*	ACY503 *glc8Δ* crossed with CEN.PK113-1A	This study

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 12 of 16

and ACY_503 (PE-2, MATa) its superior tolerant haploid. Non-tolerant laboratory MATα CEN.PK113-1A was used for the crossing with ACY_503 to generate hybrid ACY_503/CEN.PK113-1A, whose F_2 progeny was used in low pH assays. The main *S. cerevisiae* strains used in this work are presented in Table 4. *Escherichia coli* DH5α was used in cloning procedures for plasmids used in this study. Plasmid pMF002 (pMATa-EGFP-tMATa; tMATα-CyOFP-pMATα; KanMX) was used for high-throughput separation and collection of segregants using flow cytometry.

Growth media
YPD medium (10 g/L yeast extract; 20 g/L peptone; 20 g/L D-glucose), solidified with 15 g/L agar when required, was used for yeast propagation. G418 (200 μg/mL), hygromycin B (300 mg/ml) were added to the medium for the selection of strains with a KanMX or hphMX6 resistant marker, respectively. For stress-screening procedures, 1 M H_2SO_4 was used to adjust YPD pH before autoclaving. For sporulation procedures, 1% (m/v) KAc supplemented with a complete drop-out solution (460 mg/mL) was used. Cultivation occurred at 30 °C and 250 rpm unless otherwise noticed. Bacteria were grown in Luria–Bertani (LB) broth (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) supplemented with 100 μg/mL ampicillin at 37 °C.

General molecular biology
Yeast transformation was carried out with the PEG/LiAc method [78]. Transformation of the DH10β E. coli cells was done by the standard heat shock method. Bacterial plasmid purification was performed with a standard miniprep protocol [79]. Genomic DNA was extracted with (LiOAc)-SDS/EtOH fast protocol [80] for PCR purposes. All PCR reactions were performed with Phusion® High Fidelity DNA Polymerase following the manufacturer's instructions (NEB—New England Biolabs).

F_1 mating and sporulation
ACY503 and CEN.PK113-1A strains were matted by scratching them over an YPD plate. Ploidy of isolated colonies was checked by PCR and the diploid strain ACY503/CEN.PK113-1A was obtained. The diploid strain was further transformed with plasmid pMF_002. Sporulation was induced in conical glass tubes containing 2 mL of 1% KAc medium and 100 μL of a saturated culture of the diploid ACY503/CEN.PK113-1A expressing pMF_002. Tetrad formation was checked by microscope and when 80% of the culture has formed by tetrads, the cells were harvested and submitted to an asci lysis assay.

F_2 spore disruption and segregant collection
ACY_503/CEN.PK113-1A spore disruption was performed as follows: 250 μL of pelleted sporulated culture were resuspended in 100 μL of micromanipulation buffer (1 M sorbitol) with 1 μL of β-mercaptoethanol previously added. After, 16 μL of cells was added to a new Eppendorf tube containing 20 μL of Lyticase (0.5 mg/mL). The tube was vortexed and incubated at 30 °C during 3 h with shaking at 900 rpm. Digestion was checked by light microscopy and stopped with the addition of 100 μL of distilled water. The cell suspension was then vortexed for 2 min and centrifuged for 1 min, the supernatant was removed and the tube was sonicated twice for 1 min at level 2 (20%) and then diluted to $OD_{660} = 0.4$ in 1X PBS.

BD FACSAria flow cytometer III (BD-Bioscience) coupled with a cell sorter was used to sort 500,000 green (515/545) or orange (655/695) fluorescent cells each, using a FITC filter (488 nm). Sorted cells were added to 10 mL YPD containing 100 μg/mL ampicillin in glass tubes and grown for 6 h. Cultures were spun down for 5 min at 3,000 rpm and after supernatant removal cells were resuspended in 950 μl water. 200 μL of cell suspension were then plated in low-pH media ranging from 1.9 to 4. Plates were grown for 120 h and ploidy of the hypothetical segregants was assessed by multiplex PCR. In summary, genomic DNA from each colony was extracted using the protocol described by Looke [80]. Further, the extracted DNA was used as template for a multiplex PCR reaction using two distinct pairs of primers that anneal specifically to the MATa or MATα locus. The presence of a single band at 500 or 600 bp indicates that the analyzed colony poses a MATa or MATα locus, respectively. The presence of both bands in a single reaction indicates the presence of both loci in the same analyzed colony indicating that cells are diploids.

Colony spot assay for *Saccharomyces sp.* screening at low-pH conditions
Yeast phenotyping was performed as described by Takeshi et al. [81]. Initially, strains were grown overnight in 96-well plates containing 200 μL YPD. Plates were then vigorously shaken to disperse cells and a replicator block tool was used to inoculate strains on solid plates containing different low-pH conditions. Replicates were performed in three randomized positions to minimize technical errors. Growth of each strain was assessed by colony size captured by plate image using Gel Doc™ XR + Gel Documentation System (BIO-RAD, USA). The dimensions of all the images were set at 13.4 × 10 cm (W × L) and imaged under white Epi illumination with 0.5 s exposure time. Every colony pixel intensity was measured using ImageJ. The total pixel intensity within a circle (spot

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 13 of 16

radius = 50 pixels) surrounding each colony in the image was measured using the Plate Analysis JRU v1 plugin for ImageJ (http://research.stowers.org/imagejplugins/index.html). The average pixel intensity was determined by dividing the total pixel intensity by the area of the circle examined (7845 pixels2). Finally, relative growth is calculated as the ratio between the average pixel intensity of the strain colony growing on low-pH media and control (YPD) condition.

Preparation of DNA samples

Parental strains ACY_503 and CEN.PK113-1A, as well as 79 superior and inferior segregants pools for low pH assay were individually inoculated in 2 mL YPD medium and grown to the stationary phase at 30 °C. The genomic DNA was pooled extracted using the methods described by Pais et al. [82]. For each pool and condition, OD_{660} of each culture was individually assessed and the cells were mixed in equivalent concentrations to form a heterogeneous pool containing approximately the same cell concentration representative for each strain. DNA was extracted according to the procedures described by Ausubel et al. [83] and DNA concentration and quality were estimated with a Nanodrop 3000 UV–Vis spectrophotometer (Wilmington, DE, USA). The two pools and parental DNA were prepared for Illumina sequencing.

Pooled-segregant whole-genome sequence analysis and QTL mapping

At least 5 μg of gDNA from the superior and inferior low-pH resistance phenotype pools and parental strains were provided to Central Laboratory of High Performance Technologies in Life Sciences (LaCTAD) from the University of Campinas (UNICAMP) for whole-genome sequencing using the Illumina HiSeq 2500 platform. 2×100 paired-end reads were generated and aligned to the genome sequence of the CEN.PK113-7D reference strain [48]. Bowtie2 program version 2.3.5.1 [84] was used to align the paired-end reads of each sample against the reference genome. Alignment files were converted to BAM files using samtools software version 1.3.1 [85]. In addition, potential PCR duplicates were removed using picard version 2.23.9 command "MarkDuplicates" [86], in which if multiple read pairs have identical external coordinates, only the pair with the highest mapping quality is retained. SNP calling was performed using GATK (v4.0.12.0) base quality score recalibration, indel realignment and SNP and INDEL discovery and genotyping across the two samples (hard and low resistance pool) using standard high filtering parameters [66]. For each

pool the ploidy level was configured to 79 (number of haploid individuals in each pool).

The statistical analysis of QTLs was performed as proposed by Magwene et al. [87] using QTLseqR package version 0.7.0. A modified G statistic is calculated for each SNP based on the observed and expected allele depths and the value is smoothed using a Nadaraya–Watson, or tricube smoothing kernel. This smoothing method weights neighboring SNPs' G statistic by their relative distance from the focal SNP such that closer SNPs receive higher weights. The analysis was performed with an R package called QTLseqr [88]. In short, the command runGprimeAnalysis() was used to calculate the G statistic for each SNP. It then counts the number of SNPs within the set window bandwidth and estimates the tricube-smoothed G' and Δ(SNP-index) values of each SNP within that window. For both analyses a sliding window of 80 kb size was used to calculate G' of each SNP. The results were presented as − log(p-value) since p-values can be estimated from the null distribution of G', which assumes no QTL and provides a more direct statistical interpretation of QTL mapping.

Reciprocal hemizygosity analysis (RHA)

Hemizygous diploid strains were constructed by deleting the evaluated gene in one of the parental strains and crossing the haploid mutant with the opposite mating type parental strain. For example, to create the hemizygous diploid strain for *GAS1* allele, a knockout of the gene was performed on ACY_503 parental strain and further crossed to wild-type CEN.PK113-1A, creating the heterozygous diploid ACY_503/CEN.PK113-1A GAS1Δ/GAS1. The opposite strategy was made to obtain the heterozygous diploid strain ACY_503/CEN.PK113-1A GAS1/GAS1Δ. Genetic modifications were checked via PCR.

Acid wash treatment assay

The CEN.PK113-1A and CEN.PK113-1A *GAS1*[ACY_503] strains were cultivated overnight until reaching stationary phase (12–16 h). Further, cells were washed $3 \times$ with water to remove media and diluted on a water solution with pH 1.5 corrected by addition of H_2SO_4 3 M. Cells were kept on this solution under low agitation (50 rpm) and aliquots were taken every hour to assess cell viability. Cell viability was measured by staining an aliquot of cells (10 μL) with the same volume of Trypan Blue 0,4%. The solution was properly diluted and cells counted using a

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 14 of 16

Neubauer chamber. The dye exclusion test is based upon the concept that viable cells do not take up impermeable dyes (like Trypan Blue), but dead cells are permeable and take up the dye.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13068-021-02079-6.

Additional file 1. Phenotypic distribution of 48 JAY270 segregants growing on solid YPD containing decreasing concentrations of pH to define the Minimum Inhibitory Concentration. A. Boxplot graph containing the colony size values of 48 JAY270 segregants growing on solid YPD at different pH values (4; 3.5; 3; 2.5; 2.1). B. Images of each low pH plate containing the 48 segregants evaluated on this minimum inhibitory concentration assay. The images were captured 96 hours after plating.

Additional file 2. Spotting assay for growing at low pH (2.1) of ACY503/CEN.PK113-1A segregants belonging to the "high resistant pool" (A) and "low resistant pool" (B). Each lane represents a different isolated colony and the columns the dilution factor applied to the initial cultures before spotting.

Additional file 3. GAS1 mutation profile of 1053 distinct *Saccharomyces cerevisiae* strains.

Additional file 4. List of 41 Saccharomyces sp. strains evaluated during growth at low pH (2.5).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
The complete dataset of DNA-seq reads have been deposited in SRA under accession number PRJNA755995.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Genetics, Evolution, Microbiology, and Immunology, Institute of Biology, University of Campinas, Rua Monteiro Lobato 255, Campinas 13083-862, Brazil. [2]Molecular and Computational Biology Section, Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089-2910, USA.

References
1. Mira NP, Teixeira MC, Sá-Correia I. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. OMICS. Mary Ann Liebert, Inc.; 2010;14:525–40.
2. Piper P, Calderon CO, Hatzixanthis K, Mollapour M. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiol Microbiol Soc. 2001;147:2635–42.
3. Russell AD. Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. J Appl Bacteriol. 1991;71:191–201.
4. De MHF, Bonini BM, Thevelein J, Simões DA, Morais MA. Physiological and molecular analysis of the stress response of Saccharomyces cerevisiae imposed by strong inorganic acid with implication to industrial fermentations. J Appl Microbiol. 2010;109:116–27.
5. Basso LC, Basso TO, Rocha SN. Ethanol Production in Brazil: the industrial process and its impact on yeast fermentation. Biofuel Prod Dev Prospect. IntechOpen; 2011
6. Goldemberg J. The Brazilian biofuels industry. Biotechnol Biofuels. 2008;1:6.
7. dos Santos LV, de Barros Grassi MC, Gallardo JCM, Pirolla RAS, Calderón LL, de Carvalho-Netto OV, et al. Second-Generation Ethanol: The Need is Becoming a Reality. Ind. Biotechnol. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA ; 2016;12:40–57.
8. Klinke HB, Thomsen AB, Ahring BK. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol. 2004;66:10–26.
9. Jansen MLA, Bracher JM, Papapetridis I, Verhoeven MD, de Bruijn H, de Waal PP, et al. *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. FEMS Yeast Res. 2017;17:44.
10. Narayanan V, Schelin J, Gorwa-Grauslund M, van Niel EW, Carlquist M. Increased lignocellulosic inhibitor tolerance of *Saccharomyces cerevisiae* cell populations in early stationary phase. Biotechnol Biofuels. 2017;10:1–15.
11. Yang Q, Yang Y, Tang Y, Wang X, Chen Y, Shen W, et al. Development and characterization of acidic-pH-tolerant mutants of Zymomonas mobilis through adaptation and next-generation sequencing-based genome resequencing and RNA-Seq. Biotechnol Biofuels. 2020;13:1–17.
12. Zanin GM, Santana CC, Bon EPS, Giordano RCL, De Moraes FF, Andrietta SR, et al. Brazilian bioethanol program. Appl Biochem Biotechnol Humana Press. 2000;84–86:1147–62.
13. da Silva-Filho EA, Santos dos SKB, Resende do AM, de Morais JOF, de Morais MA, Simões DA. Yeast population dynamics of industrial fuel-ethanol fermentation process assessed by PCR-fingerprinting. Antonie Van Leeuwenhoek. 2005;88:13–23.
14. Laluce C, Schenberg ACG, Gallardo JCM, Coradello LFC, Pombeiro-Sponchiado SR. Advances and developments in strategies to improve strains of saccharomyces cerevisiae and processes to obtain the lignocellulosic ethanol—a review. Appl Biochem Biotechnol. 2012;166:1908–26.
15. Della-Bianca BE, Basso TO, Stambuk BU, Basso LC, Gombert AK. What do we know about the yeast strains from the Brazilian fuel ethanol industry? Appl Microbiol Biotechnol. 2013;97:979–91.

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 15 of 16

16. Basso LC, de Amorim HV, de Oliveira AJ, Lopes ML. Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res. 2008;8:1155–63.

17. Della-Bianca BE, Gombert AK. Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. Antonie Van Leeuwenhoek. 2013;104:1083–95.

18. Carvalho-Netto OV, Carazzolle MF, Rodrigues A, Bragança WO, Costa GGL, Argueso JL, et al. A simple and effective set of PCR-based molecular markers for the monitoring of the *Saccharomyces cerevisiae* cell population during bioethanol fermentation. J Biotechnol Elsevier. 2013;168:701–9.

19. Argueso JL, Carazzolle MF, Mieczkowski PA, Duarte FM, Netto OVC, Missawa SK, et al. Genome structure of a *Saccharomyces cerevisiae* strain widely used in bioethanol production. Genome Res. 2009;19:2258–70.

20. Della-Bianca BE, de Hulster E, Pronk JT, van Maris AJA, Gombert AK. Physiology of the fuel ethanol strain Saccharomyces cerevisiae PE-2 at low pH indicates a context-dependent performance relevant for industrial applications. FEMS Yeast Res. 2014;14:1196–205.

21. Guan N, Liu L. Microbial response to acid stress: mechanisms and applications. Appl Microbiol Biotechnol. 2019;104:51–65.

22. Brosnan MP, Donnelly D, James TC, Bond U. The stress response is repressed during fermentation in brewery strains of yeast. J Appl Microbiol. 2000;88:746–55.

23. Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol. 2000;74:25–33.

24. Guo Z, Olsson L. Physiological responses to acid stress by *Saccharomyces cerevisiae* when applying high initial cell density. FEMS Yeast Res. 2016;16:72.

25. Lam FH, Ghaderi A, Fink GR, Stephanopoulos G. Engineering alcohol tolerance in yeast. Sci Am Assoc Adv Sci. 2014;346:71–5.

26. Fernández-Niño M, Pulido S, Stefanoska D, Pérez C, González-Ramos D, van Maris AJA, et al. Identification of novel genes involved in acetic acid tolerance of Saccharomyces cerevisiae using pooled-segregant RNA sequencing. FEMS Yeast Res. 2018;18.

27. Fernandes AR, Mira NP, Vargas RC, Canelhas I, Sá-Correia I. *Saccharomyces cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes. Biochem Biophys Res Commun. 2005;337:95–103.

28. Kawahata M, Masaki K, Fujii T, Iefuji H. Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. FEMS Yeast Res FEMS Yeast Res. 2006;6:924–36.

29. Mira NP, Lourenço AB, Fernandes AR, Becker JD, Sái-Correia I. The RIM101 pathway has a role in *Saccharomyces cerevisiae* adaptive response and resistance to propionic acid and other weak acids. FEMS Yeast Res. 2009;9:202–16.

30. Mollapour M, Fong D, Balakrishnan K, Harris N, Thompson S, Schüller C, et al. Screening the yeast deletant mutant collection for hypersensitivity and hyper-resistance to sorbate, a weak organic acid food preservative. Yeast. 2004;21:927–46.

31. Schüller C, Mamnun YM, Mollapour M, Krapf G, Schuster M, Bauer BE, et al. Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae*. Mol Biol Cell Am Soc Cell Biol. 2004;15:706–20.

32. Desmoucelles C, Pinson B, Saint-Marc C, Daignan-Fornier B. Screening the yeast "Disruptome" for mutants affecting resistance to the immunosuppressive drug, mycophenolic acid. J Biol Chem J Biol Chem. 2002;277:27036–44.

33. Fernandes AR, Durão PJ, Santos PM, Sá-Correia I. Activation and significance of vacuolar H+-ATPase in *Saccharomyces cerevisiae* adaptation and resistance to the herbicide 2,4-dichlorophenoxyacetic acid. Biochem Biophys Res Commun. 2003;312:1317–24.

34. Meijnen J-P, Randazzo P, Foulquié-Moreno MR, van den Brink J, Vandecruys P, Stojiljkovic M, et al. Polygenic analysis and targeted improvement of the complex trait of high acetic acid tolerance in the yeast *Saccharomyces cerevisiae*. Biotechnol Biofuels. 2016;9:1–18.

35. Abbott DA, Suir E, van Maris AJA, Pronk JT. Physiological and transcriptional responses to high concentrations of lactic acid in anaerobic

chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol Am Soc Microbiol (ASM). 2008;74:5759–68.

36. Kapteyn JC, Ter Riet B, Vink E, Blad S, De Nobel H, Van Den Ende H, et al. Low external pH induces HOG1-dependent changes in the organization of the *Saccharomyces cerevisiae* cell wall. Mol Microbiol Mol Microbiol. 2001;39:469–80.

37. Mira NP, Palma M, Guerreiro JF, Sá-Correia I. Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. Microb Cell Factories. 2010;9:1–13.

38. Mira NP, Becker JD, Sá-Correia I. Genomic expression program involving the Haa1p-regulon in *Saccharomyces cerevisiae* response to acetic acid. https://home.liebertpub.com/omi. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA; 2010;14:587–601.

39. Dong Y, Hu J, Fan L, Chen Q. RNA-Seq-based transcriptomic and metabolomic analysis reveal stress responses and programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. Sci Rep. 2017;7:1–16.

40. Ribeiro RA, Vitorino MV, Godinho CP, Bourbon-Melo N, Robalo TT, Fernandes F, et al. Yeast adaptive response to acetic acid stress involves structural alterations and increased stiffness of the cell wall. Sci Rep. 2021;11:1–9.

41. Chen AKL, Gelling C, Rogers PL, Dawes IW, Rosche B. Response of *Saccharomyces cerevisiae* to stress-free acidification. J Microbiol J Microbiol. 2009;47:1–8.

42. de Lucena RM, Elsztein C, de Barros Pita W, de Souza RB, de Sá Leitão Paiva Júnior S, de Morais Junior MA. Transcriptomic response of *Saccharomyces cerevisiae* for its adaptation to sulphuric acid-induced stress. Antonie Van Leeuwenhoek. 2015;108:1147–60.

43. de Lucena RM, Elsztein C, Simões DA, de Morais MA. Participation of CWI, HOG and Calcineurin pathways in the tolerance of *Saccharomyces cerevisiae* to low pH by inorganic acid. J Appl Microbiol. 2012;113:629–40.

44. Lucena RM, Dolz-Edo L, Brul S, de Morais MA, Smits G. Extreme low cytosolic pH is a signal for cell survival in acid stressed yeast. Genes. 2020;11:656.

45. Claret S, Gatti X, Doignon F, Thoraval D, Crouzet M. The Rgd1p Rho GTPase-activating protein and the Mid2p cell wall sensor are required at low pH for protein kinase C pathway activation and cell survival in *Saccharomyces cerevisiae*. Eukaryot Cell Am Soc Microbiol. 2005;4:1375–86.

46. van Dijken J, Bauer J, Brambilla L, Duboc P, Francois J, Gancedo C, et al. An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microbial Technol. 2000;26:706–14.

47. Treusch S, Albert FW, Bloom JS, Kotenko IE, Kruglyak L. Genetic mapping of MAPK-mediated complex traits across *S. cerevisiae*. PLoS Genet. 2015;11:e1004913.

48. Nijkamp JF, van den Broek M, Datema E, de Kok S, Bosman L, Luttik MA, et al. De novo sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. Microb Cell Fact. 2012;11:36.

49. Cherry JM, Adler C, Ball C, Chervitz SA, Dwight SS, Hester ET, et al. SGD: Saccharomyces genome database. Nucleic Acids Res. 1998;26:73–9.

50. Peter J, De Chiara M, Friedrich A, Yue J-X, Pflieger D, Bergström A, et al. Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. Nat. 2018 5567701. Nature. 2018;556:339–44.

51. Prado CD, Mandrujano GPL, Souza JP, Sgobbi FB, Novaes HR, da Silva JPMO, et al. Physiological characterization of a new thermotolerant yeast strain isolated during Brazilian ethanol production, and its application in high-temperature fermentation. Biotechnol Biofuels. 2020;13:1–15.

52. Wilkening S, Lin G, Fritsch ES, Tekkedil MM, Anders S, Kuehn R, et al. An evaluation of high-throughput approaches to QTL mapping in *Saccharomyces cerevisiae*. Genetics. 2014;196:853–65.

53. Liti G, Louis EJ. Advances in quantitative trait analysis in yeast. PLoS Genet. 2012;8:e1002912.

54. Ehrenreich IM, Torabi N, Jia Y, Kent J, Martis S, Shapiro JA, et al. Dissection of genetically complex traits with extremely large pools of yeast segregants. Nature. 2010;464:1039–42.

55. Meijnen J-P, Randazzo P, Foulquié-Moreno MR, van den Brink J, Vandecruys P, Stojiljkovic M, et al. Polygenic analysis and targeted improvement of the complex trait of high acetic acid tolerance in the yeast *Saccharomyces cerevisiae*. Biotechnol Biofuels. 2016;9:5.

Coradini *et al. Biotechnology for Biofuels*   (2021) 14:239

Page 16 of 16

56. De Carvalho BT, Holt S, Souffriau B, Brandão RL, Foulquié-Moreno MR, Theveleina JM. Identification of novel alleles conferring superior production of rose flavor phenylethyl acetate using polygenic analysis in yeast. MBio. 2017;8.

57. Wang Z, Qi Q, Lin Y, Guo Y, Liu Y, Wang Q. QTL analysis reveals genomic variants linked to high-temperature fermentation performance in the industrial yeast. Biotechnol Biofuels. 2019;12:1–18.

58. Swinnen S, Schaerlaekens K, Pais T, Claesen J, Hubmann G, Yang Y, et al. Identification of novel causative genes determining the complex trait of high ethanol tolerance in yeast using pooled-segregant whole-genome sequence analysis. Genome Res. 2012;22:975–84.

59. Cubillos FA, Brice C, Molinet J, Tisné S, Abarca V, Tapia SM, et al. Identification of nitrogen consumption genetic variants in yeast through QTL mapping and bulk segregant RNA-Seq analyses. G3 Genes Genomes Genetics. 2017;7:1693–705.

60. Brice C, Sanchez I, Bigey F, Legras JL, Blondin B. A genetic approach of wine yeast fermentation capacity in nitrogen-starvation reveals the key role of nitrogen signaling. BMC Genomics. 2014;15.

61. Eder M, Sanchez I, Brice C, Camarasa C, Legras J-L, Dequin S. QTL mapping of volatile compound production in *Saccharomyces cerevisiae* during alcoholic fermentation. BMC Genomics. 2018;19:166.

62. Hubmann G, Mathé L, Foulquié-Moreno MR, Duitama J, Nevoigt E, Thevelein JM. Identification of multiple interacting alleles conferring low glycerol and high ethanol yield in *Saccharomyces cerevisiae* ethanolic fermentation. Biotechnol Biofuels. 2013;6.

63. Feng L, Jia H, Qin Y, Song Y, Tao S, Liu Y. Rapid identification of major QTLS associated with near-freezing temperature tolerance in *Saccharomyces cerevisiae*. Front Microbiol Front. 2018;9:2110.

64. Michelmore RW, Paran I, Kesseli RV. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci. 1991;88:9828–32.

65. Klis FM, Mol P, Hellingwerf K, Brul S. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol Rev. 2002;26:239–56.

66. Ragni E, Fontaine T, Gissi C, Latgè JP, Popolo L. The Gas family of proteins of *Saccharomyces cerevisiae*: characterization and evolutionary analysis. Yeast. 2007;24:297–308.

67. Carotti C, Ragni E, Palomares O, Fontaine T, Tedeschi G, Rodríguez R, et al. Characterization of recombinant forms of the yeast Gas1 protein and identification of residues essential for glucanosyltransferase activity and folding. Eur J Biochem. 2004;271:3635–45.

68. Matsushika A, Negi K, Suzuki T, Goshima T, Hoshino T. Identification and characterization of a novel Issatchenkia orientalis GPI-anchored protein, IoGas1, required for resistance to low pH and salt stress. In: Yang S, editor. PLoS One. 2016;11:e0161888.

69. Ariño J. Integrative Responses to High pH Stress in S. cerevisiae. https://home.liebertpub.com/omi. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA ; 2010;14:517–23.

70. Hunt RC, Simhadri VL, Iandoli M, Sauna ZE, Kimchi-Sarfaty C. Exposing synonymous mutations. Trends Genet. 2014;30:308–21.

71. Kristofich J, Morgenthaler AB, Kinney WR, Ebmeier CC, Snyder DJ, Old WM, et al. Synonymous mutations make dramatic contributions to fitness when growth is limited by a weak-link enzyme. PLOS Genet. 2018;14:e1007615.

72. Walsh IM, Bowman MA, Santarriaga IFS, Rodriguez A, Clark PL. Synonymous codon substitutions perturb cotranslational protein folding in vivo and impair cell fitness. Proc Natl Acad Sci. 2020;117:3528–34.

73. Fuchs BB, Mylonakis E. Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot Cell. 2009;8:1616–25.

74. Sanz AB, García R, Rodríguez-Peña JM, Arroyo J. The CWI pathway: regulation of the transcriptional adaptive response to cell wall stress in yeast. J Fungi. 2018;4:1.

75. García R, Pulido V, Orellana-Muñoz S, Nombela C, de Vázquez Aldana CR, Rodríguez-Peña JM, et al. Signalling through the yeast MAPK cell wall integrity pathway controls P-body assembly upon cell wall stress. Sci Reports. 2019;9:1–13.

76. Lesage G, Shapiro J, Specht CA, Sdicu A-M, Ménard P, Hussein S, et al. An interactional network of genes involved in chitin synthesis in *Saccharomyces cerevisiae*. BMC Genet. 2005;6:8.

77. Matsushika A, Suzuki T, Goshima T, Hoshino T. Evaluation of *Saccharomyces cerevisiae* GAS1 with respect to its involvement in tolerance to low pH and salt stress. J Biosci Bioeng Elsevier. 2017;124:164–70.

78. Gietz RD, Woods RA. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. 2002;350:87–96.

79. Engebrecht J, Brent R, Kaderbhai MA. Minipreps of plasmid DNA. Curr Protoc Mol Biol. 1991;15:1.

80. Lõoke M, Kristjuhan K, Kristjuhan A. Extraction of genomic DNA from yeasts for PCR-based applications. Bio Tech. 2018;50:325–8. https://doi.org/10.2144/000113672.

81. Matsui T, Ehrenreich IM, Mackay T, Stone E, Ayroles J, Baye T, et al. Gene-environment interactions in stress response contribute additively to a genotype-environment interaction. In: Gibson G, editor. PLOS Genet. 2016;12:e1006158.

82. Pais TM, Foulquié-Moreno MR, Thevelein JM. QTL mapping by pooled-segregant whole-genome sequencing in yeast. Methods Mol Biol. 2014;1152:251–66.

83. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith AJ, et al. Current protocols in molecular biology. USA: Wiley; 1998.

84. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357–9.

85. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078–9.

86. Picard Tools—By Broad Institute [Internet]. [cited 2021 Aug 15]. Available from: https://broadinstitute.github.io/picard/.

87. Magwene PM, Willis JH, Kelly JK. The statistics of bulk segregant analysis using next generation sequencing. In: Siepel A, editor. PLoS Comput 2011;7:e1002255.

88. Mansfeld BN, Grumet R. QTLseqr: An R package for bulk segregant analysis with next-generation sequencing. Plant Genome. 2018;11:0.

Publisher's Note