Development, Manufacturing and Fermentation Performance of Novel Hybrid Yeast Strains in Industrial Ethanol Fermentations.

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Abstract

Ethanol fermentation processes are functionally dependent on biocatalytic activity of living microorganism, *Saccharomyces cerevisiae* (yeast). Development of novel stable yeast strains with stable genotypes, resistant to stress and sometimes possessing additional metabolic activities beside central metabolism pathways involved in ethanol metabolism bring additional benefits when employed in the industrial setting.

Critically, genetically stable yeast strains with improved tolerances to the industrially relevant stress factors allow for yeast recycling without compromising their metabolic capabilities. While in some processes use of genetically modified organism is permitted significant need remains for implementing non genetically modified microorganism.

Implementation of common classical hybridization methods with robust screening allows for selection of hybrids with targeted phenotypes. When combined with genotyping strain differences can be further characterized.

To obtain new families of strains the classical hybridizations methods such as: direct mating, rare mating, and mass mating were used. Combining these hybridization methods with targeted stress focused hybrid screening allowed for selection of hybrids with improve ethanol performance characteristics vs. parental strains. In our case we have obtained genetically stable yeast strains allowing for yeast recycling, improved stress tolerance strains as well as strains with extracellular enzymatic activity. The terminal strain GSYE-GA with its STA1 activity which allows for decrease of commercial glucoamylase (GA) enzyme products use provided for allowing GA reduction in the range of 20-30 % vs. control.

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Keyword:

ethanol, fermentation, propagation, yeast, *Saccharomyces cerevisiae*, hybrid strains, high density yeast cultivation, bioprocess, cultivation, inoculum, Active Dry Yeast, ADY, biomass, bacterial, contamination, yield, efficiency, volumetric productivity, substrate, carbohydrates, dextrose, lactic acid, acetic acid, glycerol

Introduction

Improving industrial yeast strains by increasing their genetic stability and stress tolerance by using specific molecular breeding techniques to generate industrially critical yeast phenotypes that may include desired enzymatic activity allowing for complex carbohydrate hydrolysis is critically important as it allows for improved economic viability of the existing industrial ethanol manufacturing processes.

The existing industrial yeast strains were originally based on the natural yeast strains but over generations of use and selection have accumulated sometimes deleterious genome alternations resulting in increased genomic instability often manifested in their inability to sporulate or frequent changes in phenotype during yeast biomass manufacturing or terminal use of biomass products. Development of stable germline *Saccharomyces cerevisiae* haploid (single set of chromosomes, alpha and haploid lambda) or diploid state (double set of chromosomes, a/α, sporulation able) type of industrial strains adopted to multiple combined stress factors is of interest. Adaptability to stress factors such as temperature, pH and ethanol tolerance in liquid and solid-state industrial application model systems combined with higher metabolic activity, phenotype stability and predictive performance due to genome stabilization is thought to result in better performance of yeast dependent industrial processes. These targeted processes include food ingredient processing, baking applications, fermented beverages applications and commodity biochemical manufacturing such as ethyl alcohol. In addition, incorporation of targeted enzymatic hydrolytic activities for grain complex carbohydrate hydrolysis, protein matrix modifications and phytic acid degradation may be combined and introduce in the selected genetically stable industrial *Saccharomyces cerevisiae* yeast strains.

By definition normal natural yeast strains exist either in most stable haploid (single set of chromosomes) heterothallic state either as a-mating or α- mating type or diploid state (double set of chromosomes) a/α sporulation able type strain. Laboratory strains are most often haploid genetically stable parental a or α mating type strains. In difference, the industrial strains are often genetically unstable diploid, triploid, polyploid and most often aneuploid state.

Principal reference laboratory yeast strain used in yeast genetics research is strain designated as S288c, this strain only requires biotin, nitrogen source, glucose and an assortment of salts and trace elements for growth (Mortimer et al. 1984). Furthermore, strain S288c was used to derive most of nutritional mutants currently used in yeast genetics studies. It is also a reference yeast genome strain originally sequenced in 1996 with updated sequence published in 2010.

Comparison of laboratory and industrial strains reveals that later are very much so are genetically unstable. Yet since these industrial strains contain the desirable phenotypic characteristics and are typically very metabolically active, they find diverse industrial applications across different processes and product applications.

In addition, these currently significant industrial yeast strains because of their genetic instability are not typically susceptible to additional, more targeted breeding involving sporulation or molecular modifications where high genetic stability is required. In addition to the nuclear DNA instability extrachromosomal DNA such as mitochondrial DNA shows additional instabilities and often results in undesirable phenotypes. Overall, review of the current microbial and molecular biology technology status in view of the fundamental issues with genetic stability of the current industrial yeast strains, reveals that new yeast strains should be evolved from either natural or existing laboratory strains or perhaps even through genome renewal of the existing industrial yeast strains.

The genotypes of 157 mostly industrial yeast strains currently used in the beers, wine, distilled spirits and biofuels were recently describe (Gallone et al., 2016). The Phylogeny and Population Structure of these industrial S. cerevisiae strains revealed significant difference in their origins and structure but only five sublineages that are genetically and phenotypically separated from wild strains were proposed. The chromosomal copy-number variation (CNV) profiles, revealed many nominal ploidy (n) values alternations in addition to many amplification or deletion of genomic fragments. Similar studies as the one cited above revealed that additional work should be undertaken to obtain stable yeast strains.

While the genetic stability of the baseline strain is critically important since it allows for introduction of additional natural and transgenic traits. *Saccharomyces cerevisiae var. diastaticus* strains can produce an extracellular glucoamylase that enables fermentation of starch and oligosaccharides. Extracellular glucoamylase in diastatic *S. cerevisiae* is coded for by the STA1 gene which appears to be chimeric and consists of rearranged gene fragments from both FLO11 and SGA1 genes.

The goal of the project was to produce four strains that were genetically stable, poses enhance stress characteristics and had extracellular glucoamylase like activity. The strains were designated as GSY genetically stable yeast.

The strain GSYE was the strain developed to have enhanced ethanol tolerance while also being genetically stable this strain has been shown to allow for production of up to 160 g/L of ethanol. The strain GSYES was the strain that has similar characteristics to GSYE but has increased stress tolerance, temperature tolerance of 39 deg. C. The strain GSYES-GA was the strain that was developed as the hybrid of strain GSYES and the natural targeted strain of *Saccharomyces cerevisiae var. diastaticus* and allows for decreased use of extracellular glucoamylase required for glucose release from complex carbohydrates. The last strain that was developed was a genetically modified strain of

strain GSYES with chromosomally integrated transgenic genes allowing for expression of extracellular glucoamylase GSYES-GMGA.

In our project we have developed strains through use of the following general procedures that allow for sporulation of natural and industrial strains (Figure 1) and quantification and molecular characterization of haploids an diplois using: PCR mating type identification, PCR identification of targeted gene and ploidy determination using fluorescence and flow cytometry. Tetrad dissection using MSM400 Microscope (Figure 2.). The targeted isolated haploids were then subject to selection and either colony or direct mating (Figure 3.) followed by the stress challenge growth and performance evaluations (Figure 4 and 5).

Methods

The following methods were development or adapted during the project:

YEAST SPORULATION PROCEDURES

The yeast sporulation procedures that allow for sporulation of strains that will normally not sporulate were developed. In summary this sporulation technique is presented in Figure 1. Prior to sporulation three stage yeast propagations and screening assays are performed. After 3rd stage propagation the yeast population is split and two yeast sporulation mediums SPM1 and SPM 4 are used to induce the cells to sporulate.

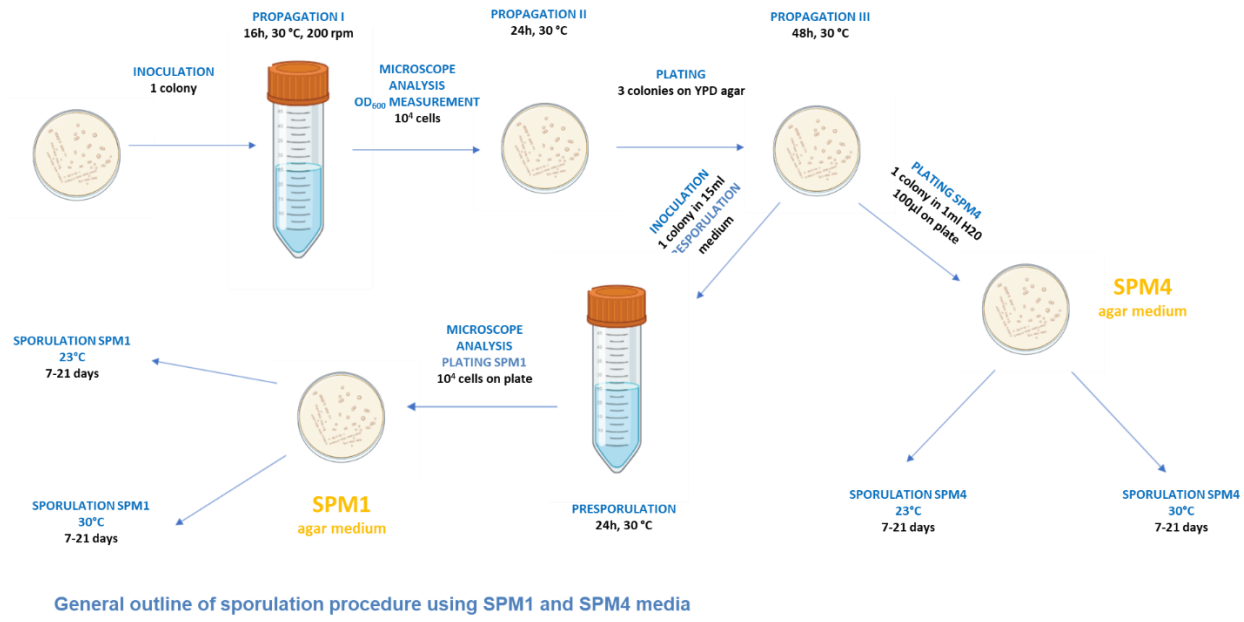


Figure 1 General Yeast Sporulation Procedure

TETRAD DISSECTION

Tetrad dissection was performed using adapted standard procedures developed for MSM400 yeast spore dissection microscope.

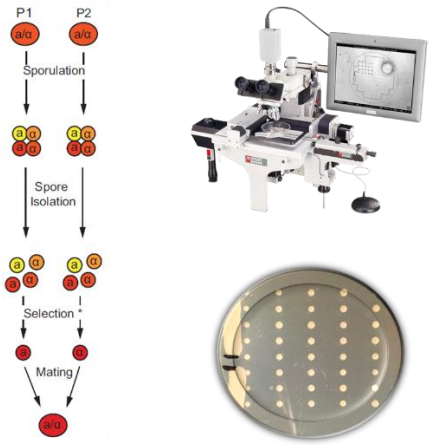


Figure 2 Tetrad Dissection, Spore Isolation, Selection and Mating.

PCR MATING TYPE IDENTIFICATION OF SACCHAROMYCES CEREVISIAE.

The Polymerase Chain Reaction (PCR) method enabling the rapid detection of MAT locus was used to allow for identification of the tested strain mating type. The primers used in the reaction: Sc_MATa_F1 : 5'-ACTCCACTTCAAGTAAGAGTTTG-3'; Sc MATα_F1 : 5'-GCACGGAATATGGGACTACTTCG-3' and Sc_MAT_R1 : 5'-AGTCACATCAAGATCGTITATGG-3'.

PCR IDENTIFICATION OF STA1 GENE.

The PCR method was used for detection of the STA1 gene. The expected PCR product size for STA1 gene and its promoter is 4017 bp. The primers used in the reaction were: BGW_532F GGAATACCGGATTGTGTGCCTAC, and BGW_533R CGAACACGCTTTGGACATCATC. Example of the PCR gel is shown below in Figure 3.

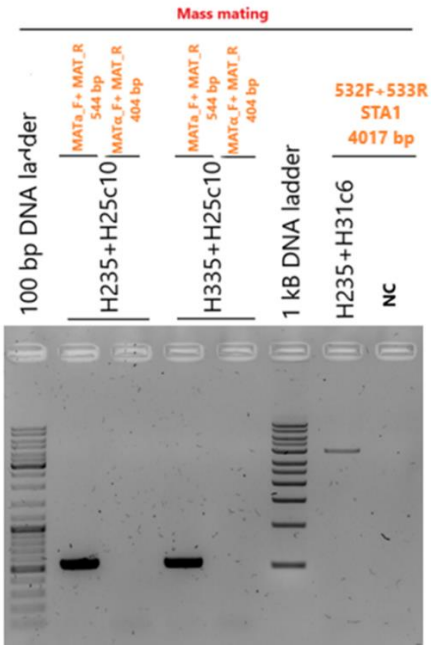


Figure 3 The PCR example for the STA1 gene product and promoter.

ACTIVITY ASSAY STA1 GENE USING SELECTIVE MEDIUM WITH COLOR CHANGE (STARCH WITH IODINE STAINING, YPS).

The assay was adopted and used for determination of hydrolytic activity of the targeted parental strains or derived hybrids. It uses selective medium subjected to color change upon starch hydrolysis.

DIRECT MATING AND MASS MATING COMBINED WITH SELECTIVE STRESS TESTING (ETHANOL AND TEMPERATURE).

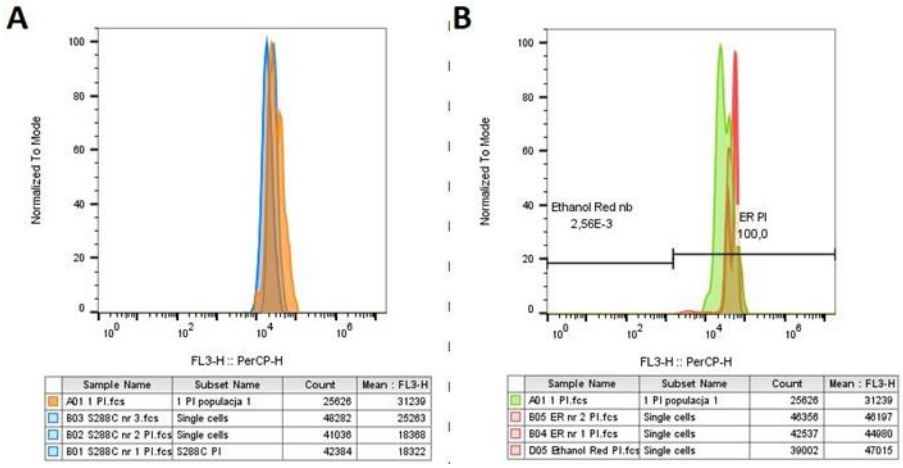
Direst and Mass mating was conducted with suspensions of haploid cells. Afterwards the resulting colonies suspensions are tested at either an increased temperature of 39 deg. C or in the presence of 80 g/L ethanol. The suspensions are transferred after 24 hours to the reversed conditions and are subjected to testing. Surviving colonies are tested in fermentation. The complete scheme is shown in figures 5 and 6. The cells suspensions were quantified using growth as determined by OD_{600} measurements.

STRAIN IDENTIFICATION USING PFGE-CHEF MAPPER SYSTEM.

Pulsed field gel electrophoresis (PFGE) was used to separate Saccharomyces cerevisiae chromosomes, Chromosomes were subjected to restrictive digestion staining using SYBR Green. The method was used for quantification of chromosomal differences.

STRAIN VIABILITY QUANTIFICATION USING FLOW CYTOMETRY.

Ploidy determination using fluorescence and flow cytometry. Propidium iodide (PI) was used as a fluorescent agent that binds DNA and RNA. RNase ensures that the RNA is removed from the sample and only stains the DNA.



Sample Name	Subset Name	Count	Mean : FL3-H
A01 1 PI.fcs	1 PI populacja 1	25626	31239
B03 S288C nr 3.fcs	Single cells	48282	25263
B02 S288C nr 2 PI.fcs	Single cells	41036	18368
B01 S288C nr 1 PI.fcs	S288C PI	42384	18322

Sample Name	Subset Name	Count	Mean : FL3-H
A01 1 PI.fcs	1 PI populacja 1	25626	31239
B05 ER nr 2 PI.fcs	Single cells	46356	46197
B04 ER nr 1 PI.fcs	Single cells	42537	44080
D05 Ethanol Red PI.fcs	Single cells	39002	47015

Figure 4 Example of histograms obtained for the test sample and the haploid control (A) and the diploid control (B), respectively.

Genetic Stability Assay.

RESULTS

The genetically stable strains that were obtained in this project allowed for successful hybridizations of the natural strains as well as stable chromosomal integration of the transgenic gene fragments. Development of the GSYE and GSYES strains was focused on fermentation testing of individual haploids obtained from the parental strains. The best performers were then subjected to hybridization followed by stress challenge testing. Several different hybridization methods were used, including colony hybridization and direct hybridization. It should be noted that colony hybridization is the combination of two methods direct hybridization and mass mating. Colony hybridization was the best method overall as it produced the most diverse and best performing hybrids. In this method a colony of

the haploid parental strains after sporulation were hybridized together. The mix culture originally designated as H25, H31, H38 were used as the source material for some of the follow up experiments. The hybridization methods were used to obtain new strains without using targeted molecular modifications.

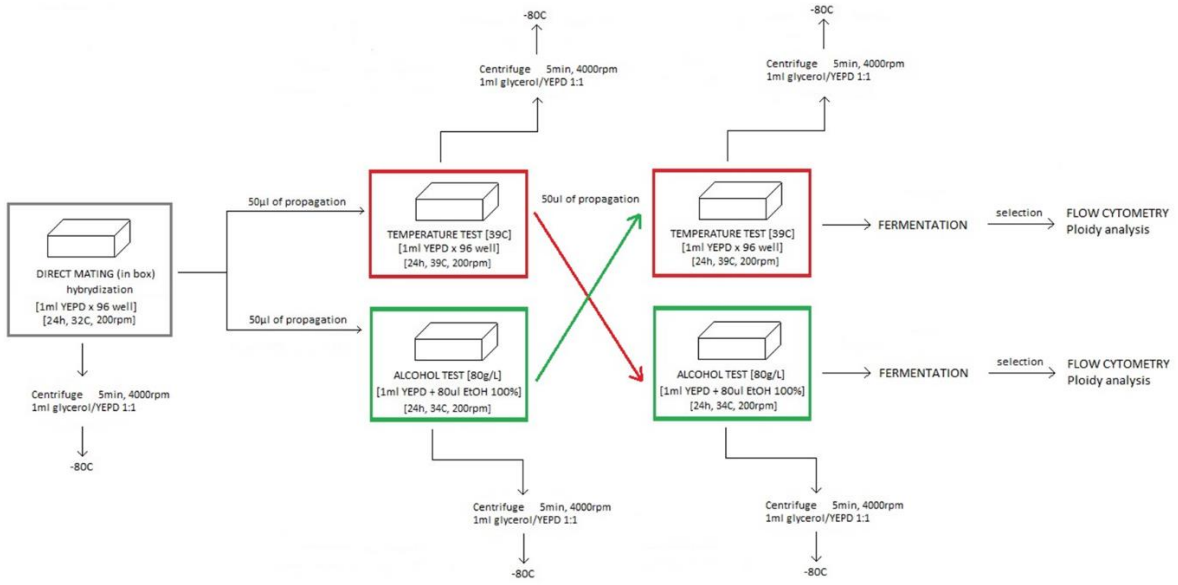


Figure 5 Direct Mating and Stress Challenge Testing for strains GSYE and GSYES.

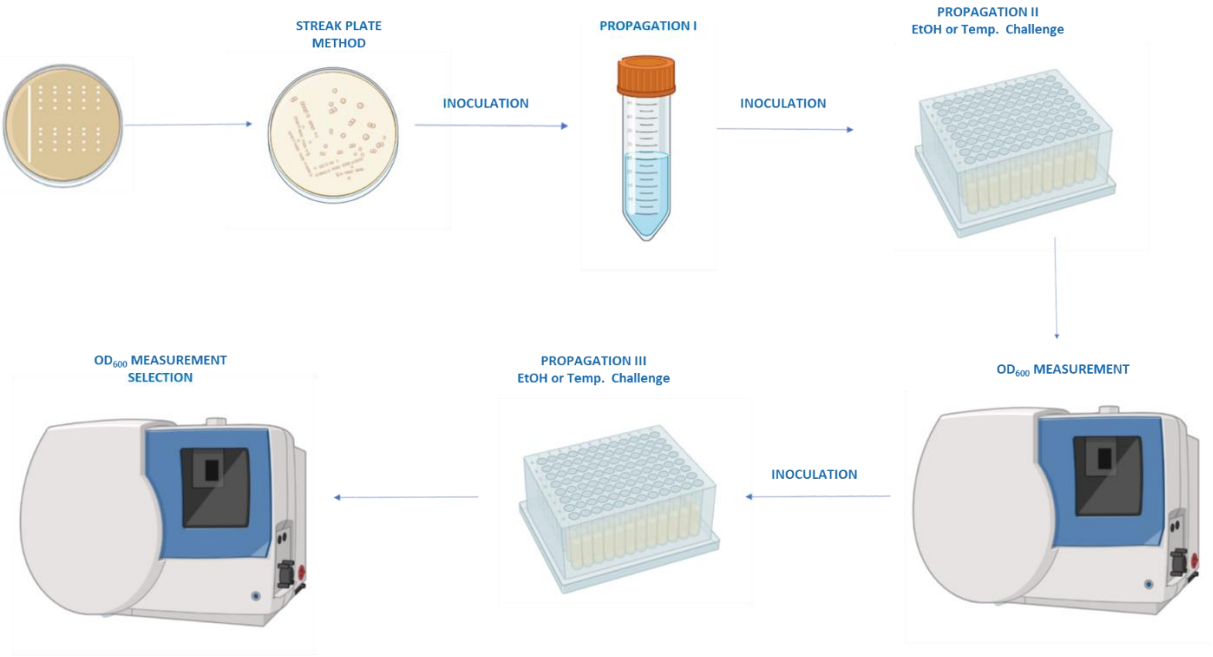


Figure 6 Quantification of Results obtained from challenge testing.

In difference the strain GSYE-GA was developed by hybridization of spores obtained from the parental GSYE or GSYES strains and targeted parental strains of Saccharomyces cerevisiae var. diastaticus H131-H135.

After several rounds of testing the 3 best performing haploids of GSYE strain were hybridized with 5 best performing haploids of Y013X strains. The resulting 355 hybrids were subjected to molecular ploidy analysis, PCR identification of STA1 gene and Activity Assay for STA1 using selective medium. Based on the above evaluation the 11 hybrid strains (H171, H235, H302, H303, H311, H335, H338, H347, H350, H352 and H355) were selected as fit for ethanol fermentation testing. In addition, each strain was sequenced using both Sanger and NGS methods. Afterwards, selected from this group H171 hybrid strain was chosen as the best candidate to move forward for hybridization with haploid strain GSY2 02 G8 designated as GSYES (Fig. 6).

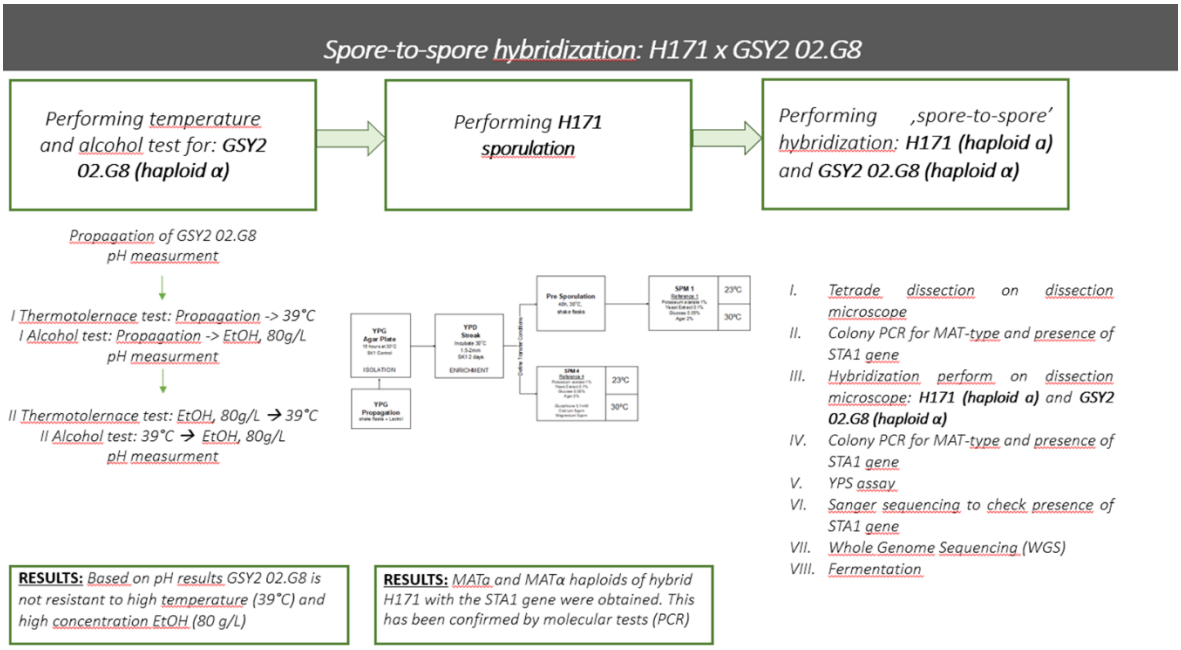


Figure 7 Direct Hybridization of Haploids H171 and GSY2 02.G8.

The resulting strains obtained from the hybridization of the H171 and GSY2 02.G8 did not however outperform the original colony hybridization strain families (H25, H31 and H38) obtained from hybridization of GSYE strains and H131-H135. Therefore, the final round of hybridization between H171 and various derivatives of H25, H31 and H38 was completed (Fig. 7).

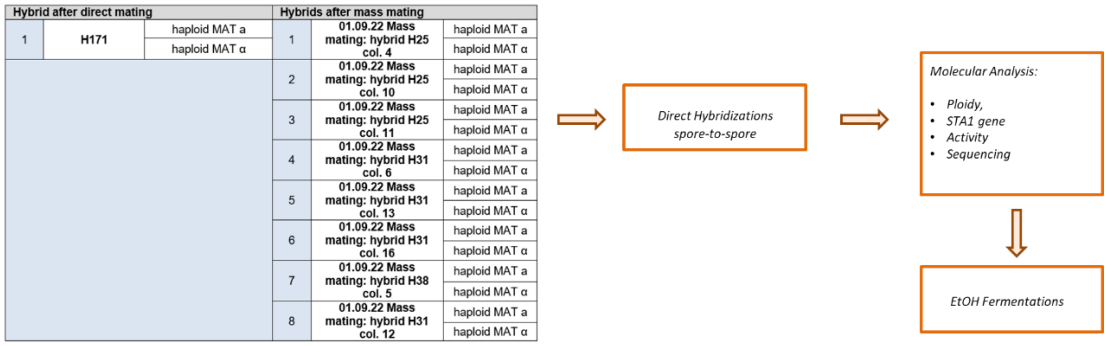


Figure 8 Direct Hybridization of Haploids H171 and H25, H31 and H38.

The final strain obtained from these hybridizations allowed for reduction of glucoamylase usage between 20-30% and increased ethanol yield in range of 2-4%, the strain was designated as GSYE-GA.

The quantification of the above strain genetic stability was performed after growth in standard medium for extended periods of time and verifying that strain could still sporulated and has maintained its phenotypic profile and its fermentation and growth performance under stress conditions was not altered.

SUMMARY

Development of novel strains using even the most basic microbiological methods is possible but must be augmented by the modern molecular biology tools. The long-term stable performance of the industrial strains is subject to natural events that may result in altered performance profiles. The summary above provides for the general overview for the work required to development of robust yeast strains. Development of the strain GSYE-GA proved to be challenging yet rewarding in view of achieving the performance targets often associated only with genetically modified microorganisms. Interestingly, based on the work conducted we believe that there are other gene targets with similar functions to that of STA1.

References:

Gallone et al., 2016, Cell 166, 1397-1410. September 8, 2016

R K Mortimer et al., 1994, Yeast 12, 1543-52. Dec 10, 1994