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Designing industrial yeasts for the consolidated bioprocessing of starchy biomass to ethanol

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Consolidated bioprocessing (CBP), which integrates enzyme production, saccharification and fermentation into a one step process, is a promising strategy for the effective ethanol production from cheap lignocellulosic and starchy materials. CBP requires a highly engineered microbial strain able to both hydrolyze biomass with enzymes produced on its own and convert the resulting simple sugars into high-titer ethanol. Recently, heterologous production of cellulose and starch-degrading enzymes has been achieved in yeast hosts, which has realized direct processing of biomass to ethanol. However, essentially all efforts aimed at the efficient heterologous expression of saccharolytic enzymes in yeast have involved laboratory strains and much of this work has to be transferred to industrial yeasts that provide the fermentation capacity and robustness desired for large scale bioethanol production. Specifically, the development of an industrial CBP amylolytic yeast would allow the one-step processing of low-cost starchy substrates into ethanol. This article gives insight in the current knowledge and achievements on bioethanol production from starchy materials with industrial engineered *S. cerevisiae* strains.

Keywords: consolidated bioprocessing, industrial yeast, codon optimization, δ-integration, raw starch, bioethanol

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Plant biomass is an abundant and renewable substrate for the sustainable production of biofuels and chemicals. The main technological hurdling the widespread conversion of this renewable resource into fuels and other valuable products is the lack of low-cost technologies to overcome the biomass recalcitrance.[1-6] Currently, bioethanol is being produced from sugarcane juice and starch-rich grains in Brazil and the United States of America using *Saccharomyces cerevisiae* strains.[7] Besides wheat and corn grains, abundant starchy feedstocks, such as wasted crop, cereal bran, potato peels and brewery spent grains, have been proposed as low-cost residual biomass for the production of bioethanol.[8-10] However, since *S. cerevisiae* lacks the amylolytic enzymes required for starch utilization, expensive enzyme addition is needed for ethanol production from starchy biomass. Thus, the industrial process of converting starch into ethanol is a costly method with four steps: milling, starch hydrolysis into glucose, yeast fermentation and alcohol distillation. Moreover, starchy materials have to be cooked (gelatinized) at a high temperature (up to 140–180°C) to obtain a high ethanol yield. To reduce the energy cost for cooking, unmodified (raw) and low temperature cooking starch fermentation systems have been proposed.[11] However, the addition of large amounts of amylolytic enzymes is still required to hydrolyze raw starch into glucose.

The cost-effective conversion of raw starch demands the expression of starch-hydrolyzing enzymes in a fermenting yeast to achieve liquefaction, hydrolysis, and fermentation (Consolidated bioprocessing, CBP) by a single organism.[12] A CBP process for raw starch conversion to ethanol can save on the excess energy demand in heating of the starch slurry, as well as

pumping or stirring of the slurry.[13-15] The CBP of raw starch would require recombinant *S. cerevisiae* strains to produce sufficient quantities of raw starch degrading enzymes to ensure hydrolysis at a high substrate loading and at moderate temperatures. This has become the primary focus of several research groups in recent years and good progress toward proof of concept has been made.[15] However, for the efficient industrial ethanol production from starch, implementing the raw starch conversion technology into robust, industrially used yeasts is crucial. Laboratory strains are easily genetically modified and the functionality of starch hydrolyzing enzymes has been proved by various laboratories.[15,16] Episomal plasmid vectors under selection have been mainly utilized for the overexpression of target genes in laboratory *S. cerevisiae* strains to ensure high copy numbers (50–200 copies) are maintained. Despite these advantages, most industrial yeasts are much more robust than laboratory strains and display higher specific ethanol productivities and ethanol yields, producing a lower amount of undesirable by-products, like glycerol.[5] Although industrial strains are particularly stable in a variety of manufacturing conditions including drying and long-term storage, their genetic engineering is challenging and, under non-selective pressure such as long run industrial processes, the usage of plasmids is undesirable as their maintenance depends on selectable markers.[17] Therefore, all genetic manipulations successfully demonstrated into laboratory strains must be transferred to industrial yeasts relying on a stable integration into the genome. Consequently, gene deletions or insertions have to be performed for all alleles to obtain a stable genotype.

The engineering of industrial *S. cerevisiae* strains can be achieved through the integration of foreign genes into their chromosomes by homologous recombination to ensure mitotic stability under non-selective conditions. The reiterated DNA sequences such as δ-sequences of the Ty retrotransposon and rDNA have been efficiently proposed as target sites which results in a greater number of integrated gene copies, and therefore higher expression levels.[18,19]

This approach has been recently assessed for the first time by integrating a glucoamylase gene into an industrial *S. cerevisiae* yeast, resulting in a promising and improvable CBP amylolytic yeast, capable of efficiently converting raw starch into ethanol.[20]

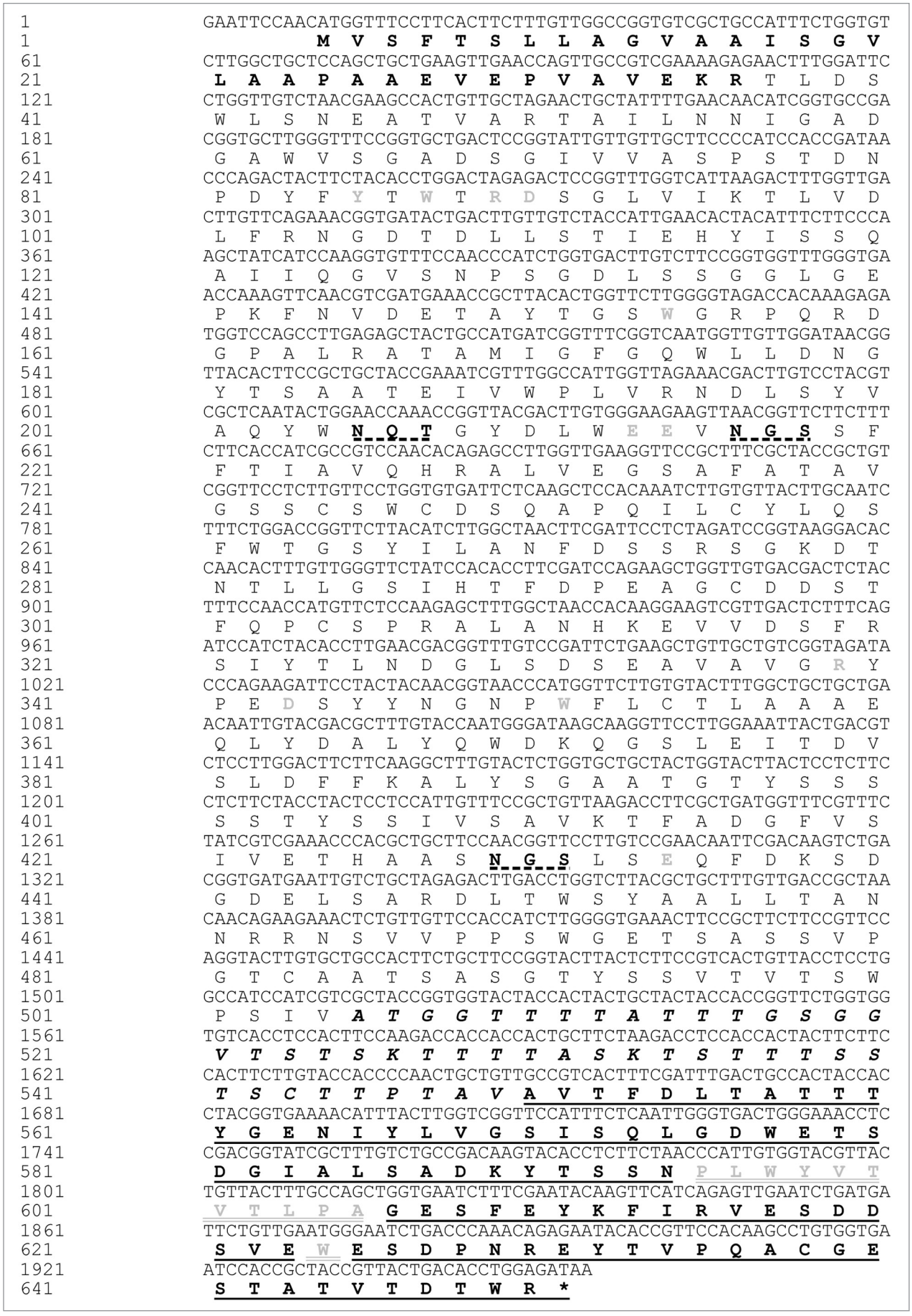
To achieve this purpose, several fungal strains of *Aspergillus oryzae* and *A. awamori*, screened for their efficient raw and soluble starch hydrolyzing activities, showed high amylolytic activities in liquid assays (data not shown). *A. awamori* CBS 115.52 was found to be a promising raw starch degrader and the cDNA copy of the glucoamylase gene *GAI* was amplified by PCR for expression in the laboratory strain *S. cerevisiae* Y294. To ensure efficient secretion of enzymes (one of the main obstacles in achieving high recombinant protein levels in engineered yeasts), the *GAI* gene was fused to the *T. reesei* β-xylanase 2 secretion signal called XYNSEC.[21] In order to further improve raw starch utilization in recombinant yeasts, the codon usage of the gene *GAI* from *A. awamori* as well as of the XYNSEC *T. reesei* β-xylanase 2 secretion signal was adapted to that of the *S. cerevisiae* but without changing the amino acid sequence (**Fig. 1**). The resulting optimized s*GAI* gene had a CAI (Codon Adaptation Index) value of 0.921 and retained conserved domains for hydrolysis of raw starch (PLWYVTVTLPA)[22] and the Gp-I region, which is heavily O-glycosylated. The glycosylation is required for both enzyme stability and enhanced activity on raw starch. Furthermore, the same Gp-I region was found to be crucial for correct folding of the enzyme.[23]

Both genes encoding the native glucoamylase from *A. awamori*, *GAI*, and the codon-optimized counterpart, s*GAI*, were cloned under the transcriptional control of the *S. cerevisiae PGK1* promoter and terminator and expressed in *S. cerevisiae* Y294 laboratory strain. The ability of the glucoamylolytic strains to produce functional recombinant enzyme was visualized as cleared hydrolysis zones in raw starch agar stained with iodine (**Fig. 2A**). The engineered yeasts were able to grow on raw starch as sole carbon source and, as reported in **Figure 2B**, the enzymatic assays clearly indicated that codon adaptation resulted in an improvement of the extracellular glucoamylolytic activity of the recombinant strains. *S. cerevisiae* Y294[ySYAG], secreting the optimized sGAI, showed a 31 and 40% increase in enzymatic activity from raw and soluble starch, respectively, compared with the enzymatic values of the native GAI, secreted by *S. cerevisiae* Y294[yASAG]. Both recombinant strains, but particularly *S. cerevisiae* Y294[ySYAG], produced limited α-amylolytic activities. Although most forms of glucoamylase can hydrolyze α-1,6-D-glucosidic bonds when the next bond in the sequence is 1,4-linked,[24] this finding is of great interest since the integration of a glucoamylase gene resulted in a recombinant yeast capable of exhibiting also a weak α-amylolytic phenotype.

Upon functional expression of both the native and synthetic glucoamylase genes into the laboratory *S. cerevisiae* Y294 strain, the *sGAI* expression cassette was integrated into the industrial *S. cerevisiae* 27P at multiple δ-sites. The resulting recombinant strains were tested for their mitotic stability using the method described in Favaro et al.[13] and the mitotically stable integrants F2 and F6 were selected as the most efficient hydrolyzing yeasts on the basis of their raw starch degradation halos (data not shown).

The glucoamylolytic activity of sGAI, tested in liquid enzymatic assays at pH values of 4.0, 4.5, 5.0, 5.5 and 6.0, was found to be optimal at pH 4.5. The raw

Figure 1 (See opposite page). Predicted protein sequence of the *sGAI* gene of *A. awamori* (GenBank:JX559863) expressed in *S. cerevisiae* Y294[ySYAG]. The *XYNSEC* secretion signal is indicated in bold font. The sequence identified in glucoamylases essential for raw starch hydrolysis was conserved (PL(W-597)YVTVTLPA),[19] as well as the second tryptophan (Trp) residue and is double underlined in gray text. The *Gp-I region* is indicated as text in italics bold. The Cp-I region or Starch Binding Domain is indicated in underlined text.[25] The general acid and base catalysts Glu-213 and Glu-434, as well as Tyr-85, Trp-87, Arg-89, Asp-90, Trp-154, Glu-214, Arg-339, Asp-343, Trp-351 sites, which play a role in substrate transition-state stabilization and/or ground-state binding, are indicated in gray text. Likely N-glycosylation sites are underlined by a broken line, although only the first and third sites were found to be glycosylated when expressed in yeast.[26]



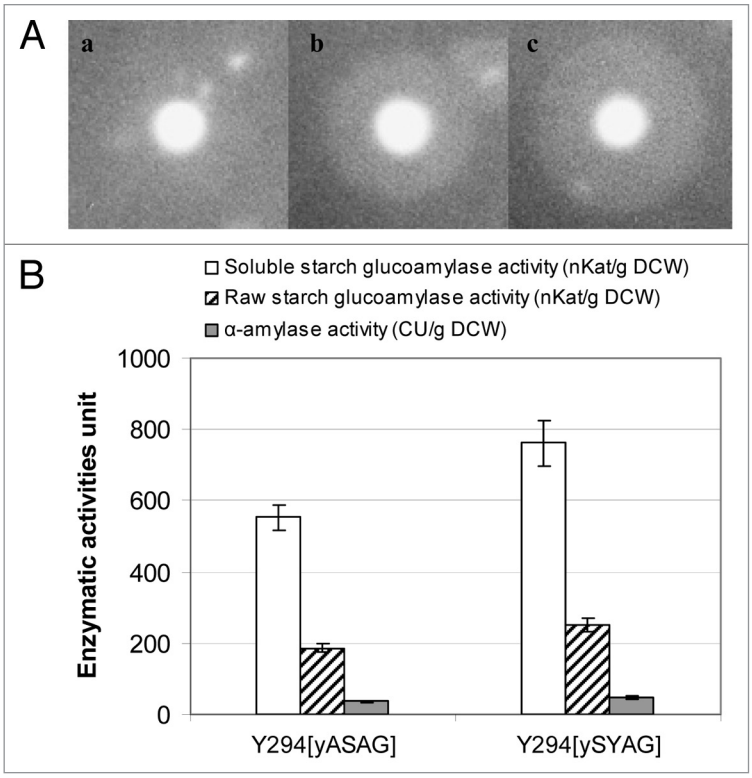


Figure 2. Glucoamylases production by wild type and recombinant *S. cerevisiae* strains. (**A**) Hydrolysis of raw starch appears as clear zones around *S. cerevisiae* colonies secreting functional glucoamylases; strain (**a**) Y294 (reference), (**b**) Y294[yASAG] secreting the native GAI, (**c**) Y294[ySYAG] secreting the codon-optimized sGAI, were grown for 4 d on agar containing raw starch and then stained with iodine solution. (**B**) Glucoamylase and α-amylase activities of the strains Y294[yASAG] and Y294[ySYAG]; glucoamylase activity, determined at pH 5.4 and 30°C, was reported as nanokatals per gram dry cell weight (nKat/g DCW), which is the enzyme activity needed to produce 1 nmol of glucose per second per gram dry cell weight. α-amylase activity, detected at pH 5.4 and 50°C, was expressed as Ceralpha Units per gram dry cell weight (CU/g DCW), which is the enzyme activity required to release 1 micromol p-nitrophenyl per min per gram dry cell weight.

and soluble starch hydrolyzing activity were assessed at different temperature values: 30, 37 and 60°C (**Table 1**). When raw corn starch was used as carbon source, the recombinant strains produced about 48% of the enzymatic activity obtained on soluble starch (**Table 1**). *S. cerevisiae* F2 displayed higher soluble and raw starch hydrolyzing activities than *S. cerevisiae* F6. This finding could be attributed to either higher number copies of *sGAI* genes integrated into the *S. cerevisiae* F2 genome compared with *S. cerevisiae* F6, or integration in regions of the genome that promotes higher transcription levels in *S. cerevisiae* F2 than in *S. cerevisiae* F6. However, further genetic studies are in progress to confirm both hypotheses. The highest enzymatic activity was detected at 60°C, while at 30°C, the growth temperature preferred by *S. cerevisiae*, the glucoamylolytic activity notably decreased to 23%. Increasing the enzymatic assay temperature up to 37°C resulted in 15% higher enzymatic values for both recombinant strains compared with 30°C.

Starch conversion was subsequently evaluated at 37°C and aerobic growth on soluble starch were more rapid than that observed at 30°C (**Fig. 3**). Both engineered yeasts were able to grow faster at 37°C than at 30°C, confirming that the higher incubation temperature positively affected the enzymatic activity of the recombinant glucoamylase (**Fig. 3B**). As a result, at 37°C, the starch to glucose conversion rate was enhanced (data not shown) and the recombinant strains reached the stationary phase earlier than at 30°C.

The recombinant *S. cerevisiae* F2 and F6 were evaluated for their ability to ferment glucose, soluble and raw starch under oxygen-limited conditions (**Table 2**). From glucose, the growth rate and the fermentative performance were comparable for all the strains. Both *S. cerevisiae* F2 and F6 showed biomass yield and ethanol production level from glucose similar to those of the parental yeast *S. cerevisiae* 27P, indicating that multiple gene integrations did not significantly affect the yeast fermentative abilities (**Table 2**). From soluble starch, the *S. cerevisiae* F2 and F6 produced high ethanol levels and their fermentative abilities were similar to those of previously engineered strains.[16,27,28] The conversion rate of starch to ethanol was found to be much more efficient in the case of *S. cerevisiae* F2 (data not shown). However, both recombinant yeasts were not able to metabolize all the starch available and the same result was observed from raw starch where the recombinants produced limited ethanol concentrations (**Table 2**). Their volumetric productivity, about 0.02 g/L/h, was much lower than those determined for previously generated strains, 0.31–0.46 g/L/h,[28] but the latter yeasts were grown in batch fermentations with higher carbon source and 25-fold larger inoculum. However, *S. cerevisiae* F2 and F6 showed the interesting ethanol yield of 75% of the theoretical, which is similar to those reported by Yamada et al.[28] As a consequence, although *S. cerevisiae* F2 and F6 secreted only the glucoamylase, their raw starch-to-ethanol conversion capability should be considered promising since the yeasts described in Yamada et al.[28] were constructed by mating two integrated haploid strains, each expressing either α-amylase or glucoamylase gene.

This study showed that codon optimization is an interesting tool for enhancing heterologous expression of genes into industrial *S. cerevisiae* strains. However, the choice of a properly selected yeast having the traits tailored for the industrial scale bioethanol process revealed to be crucial toward the design of efficient CBP amylolytic yeasts. The constructing strategy proved effective and will contribute to the high expression levels of

Table 1. Glucoamylolytic activity (nKat/DCW) of the engineered *S. cerevisiae* F2 and F6 strains once grown in YPD broth for 72 h

	Soluble starch			Raw starch		
	60°C	37°C	30°C	60°C	37°C	30°C
S. cerevisiae 27P	ND	ND	ND	ND	ND	ND
S. cerevisiae F2	3061 ± 215	833 ± 79	721 ± 56	1425 ± 90	396 ± 34	345 ± 30
S. cerevisiae F6	2380 ± 156	598 ± 48	515 ± 46	1169 ± 76	288 ± 23	251 ± 18

The assays were performed at 30 and 50°C in citrate-phosphate buffer at pH 4.5 with either 0.1% soluble starch or 2% raw starch. The values are the means of the results obtained from two experiments conducted in triplicate (± SD). ND: below detection limit.

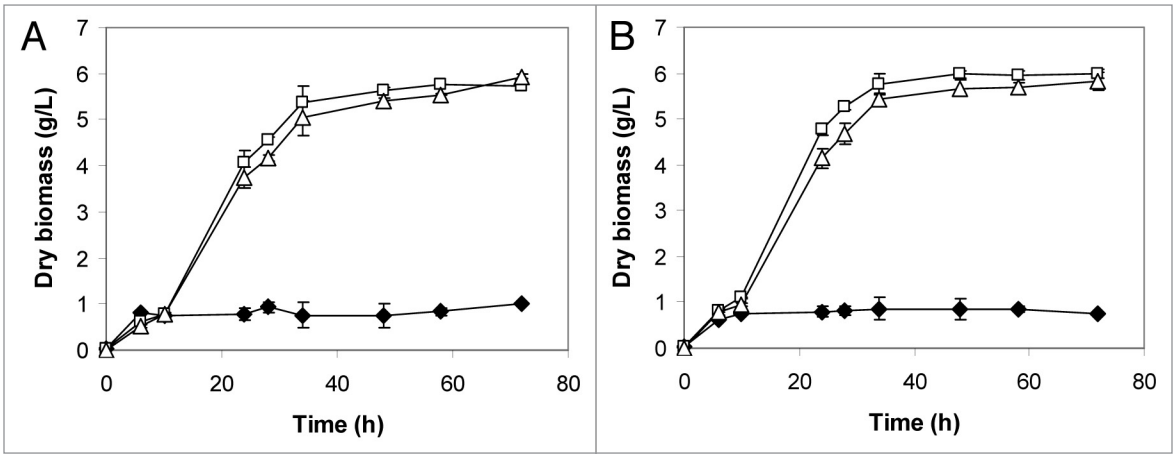


Figure 3. Effect of the temperature on the aerobic growth of *S. cerevisiae* 27P (◆), F2 (□) and F6 (△) incubated at 30°C (**A**) and 37°C (**B**) in soluble starch (20 g/L) medium.

Table 2. Ethanol production by *S. cerevisiae* strains engineered for the multiple integration of amylolytic genes

S. cerevisiae strains	Sugar[a] g/L	Ethanol g/L	*Q* (volumetric productivity) (g/L)h	Q_{max} (g/L)h	*q* (specific productivity) g(g DCW)/h	q_{max} g(g DCW)/h	Ethanol yield	Reference
Raw starch medium								
MT8–1SS	110.00	26.0 after 84 h	0.31	-	-	-	0.45 (80%)[b]	Yamada et al.[28]
NBRC1440SS	110.00	28.0 after 84 h	0.33	-	-	-	0.52 (93%)[b]	Yamada et al.[28]
MN8140SS	110.00	39.0 after 84 h	0.46	-	-	-	0.44 (79%)[b]	Yamada et al.[28]
F2	20.25	2.6 after 240 h	0.01	0.016 (48 h)	0.018	0.035 (48 h)	0.42 (75%)[b]	Favaro et al.[20]
F6	20.25	2.1 after 240 h	0.01	0.010 (48 h)	0.015	0.024 (48 h)	0.41 (74%)[b]	Favaro et al.[20]
Soluble starch medium								
SR93	55.00	14.3 after 140 h	0.10	-	-	-	0.48 (85%)[b]	Nakamura et al.[27]
F2	20.25	5.4 after 48 h	0.11	0.23 (18 h)	0.040	0.12 (18 h)	0.44 (79%)[b]	Favaro et al.[20]
F6	20.25	4.8 after 48 h	0.10	0.11 (18 h)	0.037	0.08 (18 h)	0.42 (76%)[b]	Favaro et al.[20]
Glucose medium								
27P	20.25	9.9 after 24 h	0.41	0.70 (6 h)	0.136	0.32 (6 h)	0.49 (96%)[c]	Favaro et al.[20]
F2	20.25	9.8 after 24 h	0.41	0.64 (6 h)	0.135	0.29 (6 h)	0.49 (95%)[c]	Favaro et al.[20]
F6	20.25	9.9 after 24 h	0.41	0.66 (6 h)	0.135	0.25 (6 h)	0.49 (96%)[c]	Favaro et al.[20]

[a]Sugar amounts determined from the sum of starch and glucose in medium. Ethanol yield as grams per gram of consumed sugar and percent of theoretical maximum (0.56 g/g from starch[b] or 0.51 g/g from glucose[c]) indicated in brackets. *S. cerevisiae* F2 and F6 were studied in raw starch medium (20 g/L corn starch, 6.7 g/L yeast nitrogen base, 20 g/L peptone and 0.5 g/L glucose); soluble starch medium; and glucose medium where the equivalent amount of raw starch was replaced with either soluble potato starch or glucose (Sigma).

other heterologous sequences into industrial CBP yeasts.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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