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Engineering amylolytic yeasts for industrial bioethanol production

Favaro Lorenzo[1,2], Basaglia Marina[1], Saayman Maryna[2], Rose Shaunita H.[2], van Zyl Willem H.[2] and Casella Sergio[1]

[1] Dipartimento di Biotecnologie Agrarie, Università di Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro (PD).
[2] Department of Microbiology, Stellenbosch University, Private Bag X1, 7602 Matieland, South Africa.

The development of a Consolidated Bioprocessing amylolytic yeast could yield large cost reductions in the industrial ethanol conversion of starchy substrates.
The aim of this study was to develop an efficient amylolytic *Saccharomyces cerevisiae* strain suitable for industrial bioethanol production. A wild type *S. cerevisiae* strain with promising industrial fermentative traits was engineered to secrete a fungal glucoamylase (SgaI). The obtained recombinant strains were able to hydrolyse starch and to convert the resulting glucose into ethanol.
Further preliminary fermentation studies on unmodified corn starch indicate that the engineered yeast strains could be efficiently used for the Consolidated Bioprocessing of different starchy industrial residues.

1. Introduction

The utilisation of biomass for the production of bioethanol has received considerable interest in recent years. Starchy and cellulosic materials are the most abundant biomass resources. In particular, ethanol produced from agricultural residues, industrial wastes and fast-growing plant species has been suggested to be a promising alternative fuel.
Fuel ethanol has already been produced from sugar cane and starch rich grains in Brazil and the United States. However, ethanol production from starchy materials by the conventional yeast fermentation method is a high cost process requiring the gelatination of raw starch by cooking, liquefaction by α-amylase and saccharification to glucose by glucoamylase.
These chemical processes are necessary because *S. cerevisiae* lacks the amylolytic enzymes required for starch utilisation. Therefore, to have available a raw starch hydrolysing and fermenting yeast could yield large cost reductions and improve the energy balance for starch conversion in Consolidated Bioprocessing (CBP), a one-step hydrolysis and fermentation technology (van Zyl et al., 2007).

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Very few groups have reported results on yeast strains able to utilise raw starch as carbon source (Khaw et al., 2006; Murai et al., 1998). This study aimed at developing a CBP yeast strain to be used in a bioethanol industrial plant for the conversion of raw starch and/or low cost starchy by-products.

2. Materials And Methods

2.1 Strains and media

The genotypes and sources of the plasmids, yeast and bacterial strains used in this study are summarized in Table 1. Recombinant plasmids were constructed and amplified in *Escherichia coli* XL1-Blue. The bacterial strains were cultured at 37°C on a rotating wheel in Terrific Broth or on Luria-Bertani agar (Sambrook et al., 1989). Ampicillin for selection of resistant bacteria was added to a final concentration of 100 µg/mL. *S. cerevisiae* strains were grown in YPD medium (Difco) at 30°C on a rotary shaker.

Table 1 Summary of plasmids and strains used in this study

Plasmids/Strains	Relevant genotype	Source or Reference
pBKD1	*bla*δ-sites-*TEF*$_P$-*KanMX*-*TEF*$_T$-δ-sites*	Stellenbosch Univ.
pBCF_*sgaI*	*bla* δ-sites-*PGK1*$_P$-*XYNSEC*-*sgaI*-*PGK1*$_T$ *Shble*-δ-sites	This work
ySYAG	*bla URA3 PGK1*$_P$-*XYNSEC*-*sgaI*-*PGK1*$_T$	Stellenbosch Univ.
E. coli XL1-Blue	*MRF' endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F'proAB lacq ZΔM15 Tn10(tet)]*	Stratagene (USA)
S. cerevisiae s3	*wild type with high fermentative vigour*	Favaro et al., 2008
S. cerevisiae sBCF2	*Recombinant strain with sgaI multiple copy integration*	This work
S. cerevisiae sBCF6	*Recombinant strain with sgaI multiple copy integration*	This work

* *TEF1* promoter and terminator from *Ashbya gossypii*

2.2 DNA manipulations and yeast transformation

Standard protocols were followed for DNA manipulation (Sambrook et al., 1989). Restriction endonucleases and T4 DNA polymerase were supplied by either Roche or Fermentas.

Transformation of wild type *S. cerevisiae* strain was obtained by electroporation. Delta vectors were digested with *Xho*I while yeast cells were prepared with lithium acetate procedure. After the electrotransformation (Cho et al., 1999), yeast cells were incubated in YPD supplemented with sorbitol (1M) at 30°C for 3 hours and then plated on YPD

supplemented with sorbitol and zeocin (50-100 μg/mL). After 3 days incubation (30°C), the single colonies were tested on raw starch agar (2% corn starch, 2% peptone, and 0.1% glucose), grown for 4 days at 30°C and left for 24h at 4°C. Cells expressing the amylase gene were easily recognized because they were surrounded by a clear halo due to starch hydrolysis.

2.3 Enzymatic assays

Yeast cells were aerobically grown up to 168h in YPD broth at 30°C and 5 mL samples were periodically withdrawn. After centrifugation (5000 rpm for 5 min), the supernatant was used for the assays and the dry biomass determined.

Samples of supernatant (50 μL) were mixed with 450 μL of the substrate (2% corn starch or 0.1% potato soluble starch in a pH 4.5 citrate-phosphate buffer). Both hydrolysis reactions were carried out at 30°C for 36 minutes and at 50°C for 12 minutes. The assays were stopped by boiling in a water bath for 5 minutes. Glucose in a cooled sample was determined using the peroxidase-glucose oxidase method from a glucose assay kit (R-Biopharm).

Enzymatic activities were expressed as nanokatals per gram dry weight biomass (nkat/g DW cells), which is defined as the enzyme activity needed to produce 1 nmol of glucose per second per gram cell dry weight. All experiments were carried out in triplicate and each enzymatic assay was repeated three times.

2.4 Anaerobic cultivation

Recombinant amylolytic yeast strains were cultured in Starch Fermentation Medium (SFM) - supplemented with 2% potato soluble starch (Sigma), 0.67% yeast nitrogen base with amino acids (Difco), 2% peptone, and 0.05% glucose- and in Glucose Fermentation Medium (GFM) where the equivalent amount of soluble starch was replaced with glucose.

Precultures of *S. cerevisiae* s3 and recombinant strains sBCF2 and sBCF6 grown to stationary phase in YPD medium were used to inoculate (10% v/v) 100 mL medium in 120 mL glass serum bottles in triplicate experiments. The bottles were sealed with rubber stoppers and incubated at 30°C on a magnetic stirrer. Cell dry weight, used as growth index, was periodically monitored. Ethanol, residual glucose and starch were quantified using UV-methods (R-Biopharm).

3. Results and discussion

3.1 Plasmid and amylolytic yeast strain generation

A *δ*-integrative cassette containing the fungal gene *sgaI* and zeocin resistance gene (*Shble*) as selection marker was constructed. The *sgaI,* encoding a proficient raw starch glucoamylase, was inserted in frame with the *XYNSEC* secretion signal for the constitutive expression under the transcriptional control of the *S. cerevisiae PGK1* (Phosphoglycerate Kinase) promoter and terminator. The resulting delta vector was named pBCF_*sgaI*.

The pBCF_*sgaI* plasmid was *Xho*I digested and used for the multiple copy integration of *sgaI* into the chromosomal δ-sequences of *S. cerevisiae* s3. To study mitotic stability of the obtained mutants, the transformants with the largest starch hydrolysis halos were grown in non-selective YPD broth at 30°C for 120 generations.

The two recombinant strains sBCF2 and sBCF6 were found mitotically stable since they maintained the phenotype of resistance to zeocin and hydrolytic activity on raw starch.

3.2 Expression of *sgaI* glucoamylase in *S. cerevisiae* s3.

The glucoamylase activity was studied in culture supernatants of strains sBCF2 and sBCF6 grown in YPD broth for 168h. As expected, the *sgaI* gene fused to the *PGK1* promoter was constitutively expressed since the recombinant strains constantly showed significant enzymatic activity; highest values, reported in Table 2, were obtained after 72 hour incubation.

Table 2 Glucoamylolytic activity at 30°C and 50°C on soluble and raw starch of the engineered strains grown for 72h in YPD broth.

	Soluble starch		**Raw starch**	
S. cerevisiae strains	50°C	30°C	50°C	30°C
s3	-	-	-	-
sBCF2	2122,4 ± 245,4	624,7 ± 35,6	1040,8 ± 65,9	315,3 ± 38,1
sBCF6	1778,8 ± 122,1	489,1 ± 36,4	855,6 ± 75,4	224,4 ± 17,2

However, the enzymatic activity of recombinant strains was influenced by temperature incubation and substrate. At 30°C the glucoamylolytic activity was nearly 28% of that obtained at 50°C for the secreted glucoamylase. As expected, when unmodified corn starch was used as enzymatic substrate, the integrated strains produced about 48% of their enzymatic activity performed on soluble starch (Table 2).

3.3 Fermentation studies

All strains were grown anaerobically in GFM medium to evaluate their ethanol production using glucose as carbon source. No notable difference in ethanol production was observed between wild type strain and mutants (data not shown). All strains indeed produced up to 9.8 g/L ethanol after 24h. Their ethanol yield of about 0.49 g/g corresponded to 96% of the theoretical maximum yield from glucose. This result could indicate that gene integrations targeted to the δ-elements did not significantly affect the fermentative performance.

The engineered strains were also used for direct ethanol fermentation from starch. The stable trasformants, sBCF2 and sBCF6, hydrolysed 69% and 63% of the soluble starch and produced 5.4 and 4.8 g/L of ethanol after 48h, respectively (Figure 1b and 1c).

The sBCF2 strain produced an ethanol yield of 0.44 g ethanol per gram of consumed starch (79% of theoretical maximum) while strain sBCF6 produced a yield of 0.42, corresponding to 76% of the theoretical yield (0.56 g/g). The maximum ethanol production rate of the sBCF2 strain (0.23 g/L/h) was approximately twofold that of the sBCF6 yeast (0.11 g/L/h).

The conversion rate of starch to ethanol was also found to be much more efficient in the case of sBCF2 (Figure 1b), especially up to 18h of fermentation. After 20h, the conversion rate of both strains decreased notably, possibly because SgaI glucoamylase could efficiently hydrolyse only α-1,4 linkages. Therefore, the secreted glucoamylase could not cleave all the bonds of amylopectin. As a result, starch remained partially undegraded.

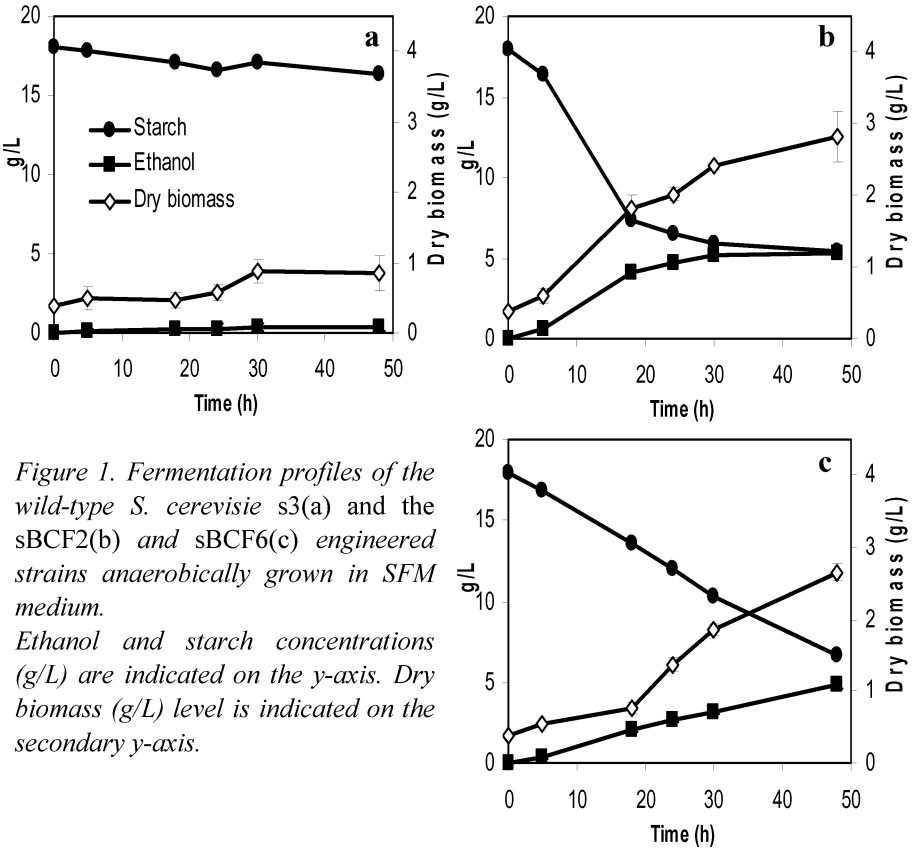

Figure 1. Fermentation profiles of the wild-type S. cerevisie s3(a) and the sBCF2(b) *and* sBCF6(c) *engineered strains anaerobically grown in SFM medium.*
Ethanol and starch concentrations (g/L) are indicated on the y-axis. Dry biomass (g/L) level is indicated on the secondary y-axis.

Since the mutant strains were able to ferment all glucose available in the medium, as reported above, the factor limiting starch-to-ethanol conversion rates seems effectively to be the inability of SgaI to hydrolyse α-1,6 linkages, usually representing a consistent fraction of bonds in the amylopectin structure (Buléon et al., 1998). The α-1,6 debranching activity of glucoamylase, indeed, could lead to complete hydrolysis of starch into glucose, improving the production of ethanol.
The co-expression of *sgaI* and proficient amylolytic genes in the constructed strains is in progress in order to enhance and optimise their starch conversion efficiency.

3.4 Conclusions

A fungal gene sequence was integrated in a wild type *S. cerevisiae* strain with optimal fermentative traits required for the industrial ethanol production. The stable

transformants secreting the SgaI enzyme showed an interesting hydrolysing activity on both soluble and raw starch and efficient ethanol production from soluble starch. Moreover, on the basis of the preliminary fermentation studies, the engineered yeast strains could be considered as promising for the Consolidated Bioprocessing of different starchy industrial residues.
The constructing strategy adopted in this work proved effective and could be applied to other bioconversion processes that use recombinant yeasts as cell biocatalyst.

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