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

Lorenzo Favaro,1 Tania Jooste,2 Marina Basaglia,1,* Shaunita H. Rose,2 Maryna Saayman,2 Johanna F. Görgens,3 Sergio Casella1 and Willem H. van Zyl2

1Department of Agrobiology, Food, Natural Resources, Animals and Environment (DAFNAE); University of Padova, Agripolis, Italy; 2Department of Microbiology; Stellenbosch University; Matieland, South Africa; 3Department of Process Engineering; University of Stellenbosch; Matieland, South Africa

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*Correspondence to: Marina Basaglia; Email: marina.basaglia@unipd.it

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 to 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.

Plant biomass is an abundant and renewable substrate for the sustainable production of biofuels and chemicals. The main technological hurdles in 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. Currently, bioethanol is being produced from sugarcane juice and starch-rich grains in Brazil and the United States of America using Saccharomyces cerevisiae strains. 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. 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. 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. 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.\textsuperscript{13-15} The engineering of industrial \textit{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 rejoinder DNA sequences such as \(8\)-sequences of the \(\delta\)-sequences of the Ty retrotransposon and \(x\)-DNA have been efficiently proposed as target sites which results in a greater number of integrated gene copies, and therefore higher expression levels.\textsuperscript{16-18}

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

To achieve this purpose, several fungal strains of \textit{Aspergillus oryzae} and \textit{A. awamori}, screened for their efficient raw and soluble starch hydrolyzing activities, showed high amylolytic activities in liquid assays (data not shown). \textit{A. awamori} CBS 115.52 was found to be a promising raw starch degrader and the \(\delta\)-DNA copy of the glucoamylase gene \(\delta\)-GAI was amplified by PCR for expression in the laboratory strain \textit{S. cerevisiae} Y294. To ensure efficient secretion of enzymes (one of the main obstacles in achieving high recombinant protein levels in engineered yeasts), the \(\delta\)-GAI gene was fused to the \(T. reesi\) \(\beta\)-xylanase 2 secretion signal called XYNSEC.\textsuperscript{21} In order to further improve raw starch utilization in recombinant yeasts, the codon usage of the gene \(\delta\)-GAI from \textit{A. awamori} as well as of the XYNSEC \(T. reesi\) \(\beta\)-xylanase 2 secretion signal was adapted to that of the \textit{S. cerevisiae} but without changing the amino acid sequence (Fig. 1). The resulting optimized \(\delta\)-GAI gene had a CAI (Codon Adaptation Index) value of 0.921 and retained conserved domains for hydrolysis of raw starch (PLWYVTFTPLA)\textsuperscript{17} and the Gp-I region, which is heavily \(\Omega\)-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.\textsuperscript{22}

Both genes encoding the native glucoamylase from \textit{A. awamori}, \(\delta\)-GAI, and the codon-optimized counterpart, \(s\)-GAI, were cloned under the transcription control of the \textit{S. cerevisiae} PFK1 promoter and terminator and expressed in \textit{S. cerevisiae} Y294 laboratory strain. The ability of the glucoamylolytic strains to produce functional recombinant enzyme was visualized as cleared hydrolysis zones in an industrial 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. \textit{S. cerevisiae} Y294(s)\(\delta\)-GAI, secreting the optimized \(s\)-GAI, showed a 31 and 40% increase in enzymatic activity from raw and soluble starch, respectively, compared with the enzymatic values of the native \(\delta\)-GAI, secreted by \textit{S. cerevisiae} Y294(s)\(\delta\)-ASAG. Both recombinant strains, but particularly \textit{S. cerevisiae} Y294(s)\(\delta\)-GAI, produced limited \(\alpha\)-amylolytic activities. Although most forms of glucoamylase can hydrolyze \(\alpha\)-1,6-glucosidic bonds when the next bond in the sequence is 1,4-linked,\textsuperscript{17} this finding is of great interest since the integration of a glucoamylase gene resulted in a recombinant yeast capable of exhibiting also a weak \(\alpha\)-amylolytic phenotype.

Upon functional expression of both the native and synthetic glucoamylase genes into the laboratory \textit{S. cerevisiae} Y294 strain, the \(\delta\)-GAI expression cassette was integrated into the industrial \textit{S. cerevisiae} Y294 at multiple \(8\)-sites. The resulting recombinant strains were tested for their mitotic stability using the method described in Favoro et al.\textsuperscript{23} and the mitotically stable integrands F2 and F6 were selected as the most efficient hydrolyzing yeast strains on the basis of their raw starch degradation halo (data not shown). The glucoamylolytic activity of \(s\)-GAI 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

\[\frac{1}{2} \text{Glucoamylase} + \text{Starch} \rightarrow \text{Reduced by-products} + \text{Ethanol} \]

Figure 1 (See opposite page). Predicted protein sequence of the \(\delta\)-GAI gene of \textit{A. awamori} (GenBank:JX559863) expressed in \textit{S. cerevisiae} Y294\(\delta\)-GAI. The XYNSEC secretion signal is indicated in bold font. The sequence identified in glucoamylases essential for raw starch hydrolysis was conserved (\textit{FourDot3}\textsuperscript{34}) and the second tryptophan (Trp) residue and is double underlined in gray text. The Gp-I region is indicated as text in italic bold. The Gp-I region is indicated in underlined text.\textsuperscript{18} The serine acceptor is a double underlined in gray text.

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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 promote 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 75% 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.26,27,28. 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 were constructed by mat- ing 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 con- tribute to the high expression levels of...
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 1. Glucoamylolytic activity (nKat/DCW) of the engineered S. cerevisiae F2 and F6 strains once grown in YPD broth for 72 h

| S. cerevisiae strains | Soluble starch | Raw starch |
|-----------------------|----------------|------------|
|                       | 60°C | 37°C | 60°C | 37°C | 60°C | 37°C | 60°C | 37°C | 60°C | 37°C | 60°C | 37°C | 60°C | 37°C |
| S. cerevisiae 27P      | ND   | ND   | 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 (± SSD). ND: below detection limit.

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

| S. cerevisiae strains | Sugar* g/L | Ethanol g/L | Q (volumetric productivity) (g/Lh) | Qmax (g/L)h | q (specific productivity) (g/g DCW)/h | q max (g/g DCW)/h | Ethanol yield | Reference |
|-----------------------|------------|-------------|-----------------------------------|------------|-------------------------------------|--------------------|-------------|-----------|
|                       |            |             |                                   |            |                                     |                    |             |           |
| Raw starch medium     |            |             |                                   |            |                                     |                    |             |           |
| M78-15S               | 110.00     | 26.0 after 84 h | 0.31                              | -          | -                                   | -                  | 0.45 (90%)  | Yamada et al. (2013) |
| NBRC 14405S           | 110.00     | 28.0 after 84 h | 0.33                              | -          | -                                   | -                  | 0.52 (93%)  | Yamada et al. (2013) |
| MN81405S              | 110.00     | 39.0 after 84 h | 0.46                              | -          | -                                   | -                  | 0.64 (79%)  | Yamada et al. (2013) |
| F2                    | 20.25      | 2.6 after 240 h | 0.01                              | 0.016 (48 h) | 0.018                                | 0.035 (48 h)       | 0.42 (75%)  | Favaro et al. (2013) |
| F6                    | 20.25      | 2.1 after 240 h | 0.01                              | 0.010 (48 h) | 0.015                                | 0.024 (48 h)       | 0.41 (74%)  | Favaro et al. (2013) |
| Soluble starch medium |            |             |                                   |            |                                     |                    |             |           |
| SJH                   | 55.00      | 14.3 after 140 h | 0.10                              | -          | -                                   | -                  | 0.48 (85%)  | Nakamura et al. (2017) |
| F2                    | 20.25      | 5.4 after 48 h | 0.11                              | 0.23 (18 h) | 0.040                                | 0.12 (18 h)        | 0.44 (79%)  | Favaro et al. (2013) |
| F6                    | 20.25      | 4.8 after 48 h | 0.10                              | 0.11 (18 h) | 0.037                                | 0.018 (18 h)       | 0.42 (76%)  | Favaro et al. (2013) |
| 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%)  | Favaro et al. (2013) |
| F2                    | 20.25      | 9.8 after 24 h | 0.41                              | 0.64 (6 h)  | 0.135                                | 0.29 (6 h)         | 0.49 (95%)  | Favaro et al. (2013) |
| F6                    | 20.25      | 9.9 after 24 h | 0.41                              | 0.66 (6 h)  | 0.135                                | 0.25 (6 h)         | 0.49 (96%)  | Favaro et al. (2013) |

*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 or 0.51 g/g from glucose) 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 hereditary sequences into indus-
trial CIP yeasts.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were
disclosed.

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References
1. Hanischek CN, Van Binsbergen G, Faru APC. Ethanol from lignocellulosic biomass: techno eco-
nomic performance in short-, middle- and long-term. Biomass Bioenergy 2006; 28:308-410. http://dx.doi.org/10.1016/j.biombioe.2005.08.020.
2. Rest M, Balat H. Recent trends in global produc-
tion and utilization of bioethanol fuel. Appl Energy 2008; 85:2727-82. http://dx.doi.org/10.1016/j.apenergy.2008.09.013.
3. Rasmund K, van Boeijen JJ, Gray VM, van Gassenrijn JW, Overkamp KM, Smits RJ, et al. Microbial cellulose-fed ethanol: a sub-
strate-oriented approach. Bioeng Bugs 2010; 1:359-71. http://dx.doi.org/10.4161/bbug.1.5.12389.
4. Ohdy M, Dinh H, Hanischek C, Balat H. Novel strategies to improve co-fermentation of pentoses with D-glucose by recombinant yeast strains in lignocellulosic hydrolysates. Bioresource 2012; 8. PMID:22893998. http://dx.doi.org/10.4183/bio-22448.
5. Soha RC, Curta MA. Ethanol production from lignocellulosic biomass by recombinant Eukaryotic cell strains. FBRB. Bioresource 2012; 8. PMID:22789543.
6. Rothack RI, Schlüter MA. Biotechnological proce-
sses for conversion of corn into ethanol. Appl Microbiol Biotechnol 2005; 67:19-29. PMID:15795973. http://dx.doi.org/10.1007/s00253-004-1184-8.
7. Kim S, Dale BM. Global potential ethanol production from natural starch and syrup residues. Biomass Bioenergy 2006; 24:161-7. http://dx.doi.org/10.1016/j.biombioe.2003.08.002.
8. Favaro L, Baugli M, van Zyl WH, Cifelli S. Using an efficient fermenting yeast enhances ethanol pro-
duction from wheat malt by Saccharomyces cerevisiae. Appl Energy 2012; 99:60-9. http://dx.doi.org/10.1016/j.apenergy.2012.08.009.
9. Favaro L, Baugli M, Cifelli S. Processing wheat beer into ethanol using solid media and highly fermentative yeasts. Biomass Bioenergy 2012; http://dx.doi.org/10.1016/biomassbioenergy.2012.07.010.
10. Fukada H, Kato A, Tanaka A, Sowemn@hotmail.com: sustainable fuels from biomass and fungal cellulosic biohydrolysates. Bioenergy 2012; 199. http://dx.doi.org/10.1016/j.energy.2011.10.001.
11. van Zyl WH, Lyrd LB, den Haan R, McBride J.E. Consolidated bioprocessing for biofuel produc-
tion using Saccharomyces cerevisiae. Adv Biochem Eng Biotechnol 2007; 108:205-39. PMID:17846720.
12. van Zyl WH, Lyrd LB, den Haan R, McBride J.E. Consolidated bioprocessing for biofuel produc-
tion using Saccharomyces cerevisiae. Adv Biochem Eng Biotechnol 2007; 108:205-39. PMID:17846720.
13. Favaro L, Baugli M, Saayman M, Rose SH, van Zyl WH, Cifelli S. Engineering amylolytic enzymes for industrial bioethanol production. Chemical Engineering Transactions 2010; 24:97-102. http://dx.doi.org/10.3303/CET1020017.
14. Favaro L, Baugli M, Tonioni A, Saayman M, Rose SH, van Zyl WH, et al. Development of raw starch hydrolyzing yeasts for industrial bioethanol produc-
tion from raw starch. Appl Microbiol Biotechnol 2012; 95:1377-88; PMID:22797599; http://dx.doi.org/10.1007/s00253-012-4248-0.
15. van Zyl WH, van Zyl WH, Bloom M, Viktor MJ. Engineering recombinant yeast having glucoamylase activity. Microbiology 2012; 158:1947-58. http://dx.doi.org/10.1099/micro.0.039038-0.
16. van Zyl WH, Bito Y, Adachi T, Tanaka T, Ogino C, Fier C, Farwick A, Benisch F, Brat D, Dietz H, Farwick A, Boles E. Novel microbial renewable feedstock utilization: a sub-
strate-oriented approach. Bioeng Bugs 2010; 1:359-71. http://dx.doi.org/10.4161/bbug.1.5.12389.
17. Storey JC, Kulaik I, Scorer CA, Clare JJ. Foreign gene expression in yeast: a review. Yeast 1992; 8:423-35. http://dx.doi.org/10.1002/yea.1863.
18. Okada H, et al. Analysis of the raw starch-binding domain by mutation of a glucoamylase fromAspergillus awamori. Carbohydrate Research 1993; 211:717-24; PMID:7679638; http://dx.doi.org.10.1016/0008-6215(93)85012-U.
19. Nakamura Y, Kawachi M, Kikutani F, Ohnaga M, Sawada T. Alcohol fermentation of starch by a genetic recombinant yeast having glucoamylase activity. Chem Biochem 1997; 24:829-35; PMID:9497685.
20. Chen MH, et al. Substrate utilization of ascospore indicative inAspergillus awamori: gluco-
amylase by new defined management to eliminate N-glycosylation and inactivation by denaturation. Biochim Biophys Acta 1999; 145; 480-9. http://dx.doi.org/10.1016/S0006-291X(98)00350-5.
21. Behbahani NJ, Williamson G. Specificity of the bind-
ing domain of glucoamylase 1. Est. Bioch Chem 1995; 21:72-7. PMID:7879306. http://dx.doi.org/10.1016/0303-9383(95)80760-X.
22. Chen MH. et al. Substrate utilization of ascospore indicative inAspergillus awamori: gluco-
amylase by new defined management to eliminate N-glycosylation and inactivation by denaturation. Biochim Biophys Acta 1999; 145:480-9. http://dx.doi.org/10.1016/S0006-291X(98)00350-5.
23. Nakamura Y, Kawachi M, Kikutani F, Ohnaga M, Sawada T. Alcohol fermentation of starch by a genetic recombinant yeast having glucoamylase activity. Chem Biochem 1997; 24:829-35; PMID:9497685.
24. van Zyl WH, Lyrd LB, den Haan R, McBride J.E. Consolidated bioprocessing for biofuel produc-
tion using Saccharomyces cerevisiae. Adv Biochem Eng Biotechnol 1997; 67:19-25; PMID:9352677. http://dx.doi.org/10.1002/yea.1863.
25. Fassan L, Baugli M, Rose SH, Tonioni A, Saayman M, van Zyl WH, et al. Integration technique and efficient heterologous expression in yeasts refined for bioethanol production. Year 2011; 2011. 3586. http://dx.doi.org/10.1007/10_2007_061.
26. Favaro L, Jonco T, Baugli M, Rose SH, Saayman M, Girgoyt JJ, et al. Codon-optimized glucoamylase eGus ofAspergillus awamori improves starch utiliza-
tion in industrial yeast. Appl Microbiol Biotechnol 2012; 95:957-68. http://dx.doi.org/10.1007/s00253-012-4001-8.
27. van Haan R, McBride J.E, La Grape DG, Lyrd LB, van Zyl WH. Functional expression of ad-
 cellulose-hydrolysing in Saccharomyces cerevisiae through a one-step conversion of cellulose to ethanol. Enzyme Microb Technol 2007; 40:1295-9. http://dx.doi.org/10.1016/j.enzmictec.2006.09.022.
28. Groe M, Saayman T, Fukushima K, Hayahida S. Analysis of the car starch-binding domain by mutation of a glucoamylase fromAspergillus awamori: car starch-binding domain inAspergillus awamori. Carbohydrate Research 1999; 333:256-66. http://dx.doi.org/10.1016/S0008-6215(99)00048-3.