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Production of Ethanol from Starch by Respiration-Deficient Recombinant *Saccharomyces cerevisiae*

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A 100%-respiration-deficient nuclear petite amylolytic *Saccharomyces cerevisiae* NPB-G strain was generated, and its employment for direct fermentation of starch into ethanol was investigated. In a comparison of ethanol fermentation performances with the parental respiration-sufficient WTPB-G strain, the NPB-G strain showed an increase of ca. 48% in both ethanol yield and ethanol productivity.

The bioconversion of starch into ethanol is a two-step process. The first step is saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as glucoamylase and α-amylase. The second step is fermentation, where sugar is converted into ethanol using *Saccharomyces cerevisiae* (9, 12). The use of amylolytic yeasts for the direct fermentation of starch is an alternative to the conventional multistage process which offers poor economic feasibility. Although there are over 150 amylolytic yeast species, their industrial use is limited because of their low ethanol tolerance (11). Therefore, most research is focused on the development of genetically engineered amylolytic strains of *S. cerevisiae*, and in these strains, heterologous genes encoding α-amylase and glucoamylase from various organisms have been expressed and their products excreted (2, 4–6, 10, 15, 16, 18).

Several studies have pointed out the potential of utilizing respiration-deficient nuclear petites for the commercial production of ethanol (8, 13). Despite the vast number of strategies adopted for the construction of amylolytic strains of *S. cerevisiae*, there have been no reports about the application of respiration-deficient nuclear petites for the production of ethanol from starch. Hence, we were interested in determining the extent of improvement that this mutation would bring to starch-utilizing ethanol fermentation processes. We report for the first time the development of a respiration-deficient nuclear petite *S. cerevisiae* strain excreting a bifunctional fusion protein that contains both *Bacillus subtilis* α-amylase and *Aspergillus awamori* glucoamylase activities.

The 100%-respiration-deficient nuclear petite FY23Δpet191 mutant of the parental haploid *S. cerevisiae* FY23 strain (*MAT***a** *ura3-52 trp*Δ*63 leu2*Δ*1*) (19) was generated using PCR-mediated disruption of the *pet191* gene with a *kanMX4* disruption cassette that determines G418 sulfate (Geneticin) resistance in yeast (8). The *S. cerevisiae* NPB-G strain was generated by transforming (17) the FY23Δpet191 strain with the pPB-G plasmid (5), which contains the *B. subtilis* α-amylase and the *A.*

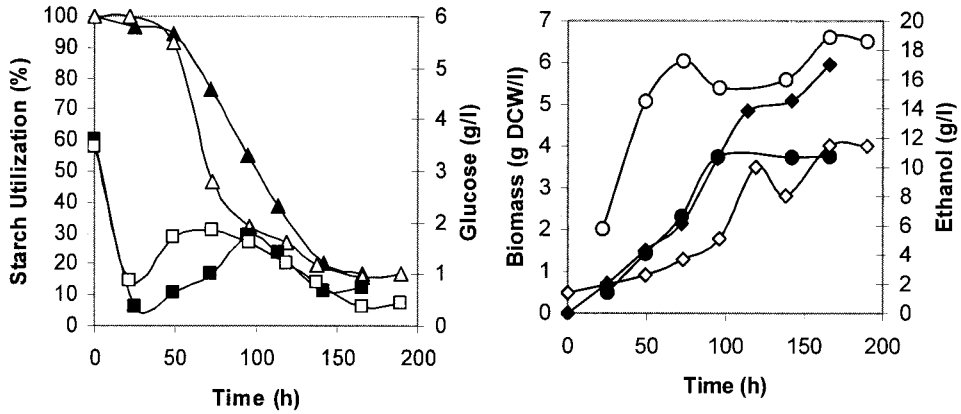

FIG. 1. Time-dependent starch utilization (▲, △) and glucose (□, ■), ethanol (◇, ◆), and biomass concentration (○, ●) profiles of NPB-G (filled symbols) and WTPB-G (open symbols) strains. DCW, dry cell weight.

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TABLE 1. Comparison of fermentation characteristics of nuclear mutant NPB-G and wild-type WTPB-G strains[a]

Medium	Strain	Biomass (g/liter)	μ_{max} (h^{-1})[b]	Maximum ethanol concn (g/liter)	$Y_{X/S}$ (g biomass/g substrate)	$Y_{P/S}$ (g ethanol/g substrate)	q_P (g ethanol/liter/h)
YEP-S + 5% starch + 0.4% (wt/vol) glucose	NPB-G	3.76	0.033 ± 0.04	17.0	0.091	0.410	0.102
	WTPB-G	6.61	0.034 ± 0.001	11.5	0.159	0.276	0.069
YE-salts + 5% starch + 0.4% (wt/vol) glucose	NPB-G	2.35	0.026 ± 0.06	8.59	0.066	0.243	0.052
	WTPB-G	3.85	0.029 ± 0.03	8.84	0.090	0.209	0.053

[a] $Y_{X/S}$, biomass yield on substrate (biomass produced [in g dry cell weight/liter] divided by the total amount of starch utilized [in g/liter]); $Y_{P/S}$, ethanol yield on substrate (ethanol produced [in g/liter] divided by the total amount of starch utilized [in g/liter]); q_P, ethanol productivity (ethanol produced [in g/liter] divided by the total fermentation time [in hours]).
[b] Results shown are means ± standard deviation.

awamori glucoamylase genes expressed under the control of the PGK1 promoter as an excreted fusion protein. Transformants were selected on yeast minimal medium-agar minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, 0.01% uracil, 0.01% tryptophan, and 0.2 mg/ml G418 sulfate. The *S. cerevisiae* WTPB-G strain was generated by transforming the parental haploid FY23 strain with the pPB-G plasmid and selecting the transformants on yeast minimal medium-agar plates without G418 sulfate. Amylolytic activity was detected by the formation of starch hydrolysis zones on plates stained with iodine. Ethanol fermentation was carried out in an orbital shaker (Innova model no. 4340) at 30°C under aerobic conditions with agitation at 180 rpm.

NPB-G mutant and WTPB-G parent cells were grown in shake flask cultures containing YEP-S medium (0.5% yeast extract, 1% peptone, 0.01% uracil, 0.01% tryptophan, 5% starch, 0.4% glucose). The concentrations of biomass, glucose, residual starch, and ethanol and the stability of the pPB-G plasmid were determined as described elsewhere (1). Figure 1 shows the time-dependent variations in starch utilization, glucose concentration, biomass formation, and ethanol production of both cultures. The ethanol fermentation characteristics of the WTPB-G strain were compared with those of the nuclear petite NPB-G strain (Table 1).

Lower biomass concentrations and biomass yields from starch were observed with the NPB-G strain as expected since the nuclear petite strain generates its energy requirements by fermentation only. Maximum specific growth rates (μ_{max}) were calculated by linear regression from ln *x*-versus-time values in exponential growth phase, with *x* being the dry cell weight. The μ_{max} of strain NPB-G in comparison with that of strain WTPB-G shows that the nuclear mutation had no significant effect on this parameter, as was also reported by Hutter and Oliver (8) for the wild-type FY23 and mutant FY23Δpet191 strains. Increases of ca. 48% in both ethanol yields and ethanol productivities from starch were achieved with the respiration-deficient NPB-G strain compared with those of the respiration-sufficient WTPB-G strain. This is in excellent agreement with the previously reported 43% increase, where the ethanol productivities of the FY23 and FY23Δpet191 strains were compared in a complex medium with glucose as the carbon source (8). The data for lower biomass and higher ethanol yields from starch as the substrate are in good agreement with observations reported earlier by Hutter and Oliver (8) and Panoutsopoulou et al. (13) for nuclear petite *S. cerevisiae* strains using glucose as the substrate and by Shi et al. (14) for a nuclear petite *Pichia stipitis* yeast utilizing xylose for ethanol production.

With both strains, about 16% of the initial starch remained unconsumed at the end of the fermentation (Fig. 1). In all experiments, the inoculum was grown in a selective medium to ensure plasmid stability. Since complex YEP-S medium does not select for the maintenance of the expression plasmid, time-dependent plasmid stability and extracellular α-amylase and glucoamylase activities determined as described by de Moraes et al. (5) were also followed in all cases. Generally, while glucoamylase and α-amylase activities varied within the range of 300 to 9,000 U/ml and 700 to 1,200 U/ml, respectively, the stability of the pPB-G plasmid was found to drop to less than 20% after 100 h of fermentation (data not shown). Improvement of expression plasmid stability in *S. cerevisiae* by decreasing the yeast extract concentration in the fermentation medium has been reported (2, 7). In order to improve the amylolytic activity of the strains, and hence to provide more efficient utilization of starch, WTPB-G and NPB-G cells were grown in YE-salts medium [0.1% yeast extract, 0.1% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% uracil, 0.01% tryptophan, 5% starch, 0.4% glucose] (3). Lowering the yeast extract in the medium resulted in lower biomass and ethanol yields with decreased μ_{max} (Table 1). Although the ethanol production yield with starch as the substrate of the petite mutant was 16% higher than that of the wild-type strain, both strains showed similar ethanol productivities with no significant improvement in plasmid stability. To use the respiration-deficient NPB-G strain for future industrial purposes, it should be improved by multiple integration of the *B. subtilis* α-amylase and the *A. awamori* glucoamylase genes into the *S. cerevisiae* FY23Δpet191 chromosome to overcome the plasmid instability.

This study constitutes a first step that provides the basis for utilizing nuclear petite mutants for the single-step bioconversion of starch into ethanol.

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