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The effect of flocculation on the efficiency of raw-starch fermentation by *Saccharomyces cerevisiae* producing the *Lipomyces kononenkoae LKA1*-encoded α-amylase

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Abstract - A major limitation of most industrially important *Saccharomyces* yeast strains are their inability to efficiently convert starch-rich substrates into commercially important commodities, such as bioethanol, low carbohydrate beer and grain whiskey. In an attempt to overcome this impediment, we have previously expressed in *Saccharomyces cerevisiae* the *LKA1* α-amylase-encoding gene from an efficient raw-starch degrading yeast, *Lipomyces kononenkoae*. Although the engineered *S. cerevisiae* strain was capable of utilising starch, the growth rate was much slower than in glucose-containing media and the ethanol yield in batch fermentations was nowhere near the levels required for an economically viable bioconversion process. The purpose of the present study was to further improve the fermentation performance of the engineered yeast by expressing the *LKA1* gene in a flocculent and non-flocculent genetic background. Despite producing similar levels of α-amylase activities in the extracellular culture media, the flocculent *S. cerevisiae* transformants degraded starch at an earlier hydrolytic window than the non-flocculent transformants. In small-scale batch fermentations, the non-flocculent strain consumed 76% of the starch supplied in the culture medium and produced 4.61 g l^{-1} of ethanol after 90 h, while the flocculent strain utilised 82% of the starch and produced 5.1 g l^{-1} of ethanol after 90 h. Flow-cell system and atomic force microscopy revealed that the 'tighter' interaction between the flocculent cells and the starch granules might contribute to the better performance of the flocculent transformant.

Key words: α-amylase, bioethanol, flocculation, *Lipomyces kononenkoae*, *Saccharomyces cerevisiae*, starch fermentation, yeast.

INTRODUCTION

The bioprocessing of starch-rich materials for the production of alternative energy sources has received considerable attention in recent years. Impediments to the effective bioconversion of starchy materials to valuable products have been the identification of organisms and biosynthetic pathways needed to produce valuable intermediates and products, and the optimisation of the organisms needed to produce the desired products with high yields. This has stimulated research into amylolytic microorganisms and their enzymes and into the broadening of the substrate range for *Saccharomyces cerevisiae* (Pretorius, 1997; Lynd *et al*., 2002). However, naturally-occurring biocatalysts in the form of organisms and enzymes are not optimally suited for industrial applications.

Saccharomyces cerevisiae has been the most utilised microorganism for industrial fermentations, but it lacks the amylolytic system to degrade starch. There are several reports on the development of recombinant *Saccharomyces* strains capable of secreting heterologous amylolytic enzymes (Steyn and Pretorius, 1991; Janse and Pretorius, 1995; Steyn and Pretorius, 1995; Steyn *et al*., 1995; Murai *et al*., 1997; Nakamura *et al*., 1997; Birol *et al*., 1998; Murai *et al*., 1999; Kondo *et al*., 2002; Shigechi *et al*., 2002; Eksteen *et al*., 2003; Shigechi *et al*., 2004; Khaw *et al*., 2006). Although a wide variety of enzymes has been successfully secreted in *S. cerevisiae*, the abilities of these enzymes to decompose starch are low because of the limited quantities of secreted enzymes in the extracellular medium. In an attempt to exploit the secreted enzymes efficiently for the production of ethanol, various strategies have been applied in starch fermentations. These include the cell surface display of amylolytic enzymes on flocculent yeast cells, the use of mixed culture fermentations, and the use of immobilised whole cell biocatalysts. Of the various strategies used, flocculation has proved to be an interesting option for ethanol production, because the time of occurrence and intensity of flocculation can affect the degree of fermentation, and consequently the alcohol lev-

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els (Masy *et al*., 1992; Seong *et al*., 2006). Flocculation is also one of the important characteristics of yeast strains used in industrial applications, such as brewing and the production of recombinant proteins, because the flocculent yeast can easily be separated during downstream processing (Kondo *et al*., 2002).

The purpose of this study was to investigate the effect of flocculation on the efficiency of raw-starch fermentation by a recombinant *S. cerevisiae* strain producing the *Lipomyces kononenkoae LKA1*-encoded α-amylase (LKA1). We have previously reported on the fermentation efficiency of *S. cerevisiae* strains producing the *LKA1* and *LKA2*-encoded α-amylases of *L. kononenkoae* LKA1 (Steyn *et al*., 1995; Eksteen *et al*., 2003). We have shown that the *LKA1*-encoded α-amylase acts on both the α-1,4 and α-1,6 linkages of the two components of starch, amylase and amylopectin (Steyn and Pretorius, 1995). Although the *S. cerevisiae* transformant producing the LKA1 enzyme was capable of utilising starch, its growth rate was much slower than in glucose-containing media and the ethanol yield in batch fermentations was nowhere near the levels required for an economically viable bioconversion process. We hypothesised that a flocculent phenotype might decrease the time interval for interaction between the enzyme and the substrate, thus facilitating substrate degradation without a time lag. To understand the effect of flocculation in this context, flocculent and non-flocculent *S. cerevisiae* strains producing the *L. kononenkoae LKA1*-encoded α-amylase were constructed. The effects of the flocculent and non-flocculent phenotypes in strains producing the LKA1 enzyme on the bioconversion of starch to ethanol in small-scale batch fermentations were examined and compared. The results obtained from these comparisons indicated that flocculation boosted the ability of the recombinant *S. cerevisiae* strain to convert starch into ethanol, thereby contributing towards laying the foundation for the possible development of efficient starch-degrading *S. cerevisiae* strains that could eventually be used in the brewing, whisky and biofuel industries.

MATERIALS AND METHODS

Recombinant DNA methods, plasmid construction and transformation. Standard procedures for isolation and manipulation of DNA were used throughout this study (Ausubel *et al*., 1995). Restriction enzymes (Roche, Mannheim, Germany), T4 DNA-ligase (Promega, Madison, WI, USA) and Takara *Ex-Taq* DNA polymerase (TakaRa Bio, Shiga, Japan) were used according to the specifications of the supplier. Bacteria were transformed as described by Ausubel *et al*. (1995), while the lithium acetate method (Gietz *et al*., 1992) was used for yeast transformations.

Microbial strains and plasmids. The sources and relevant genotypes of bacterial and yeast strains, together with the plasmids used in this study, are listed in Table 1. *Escherichia coli* strain DH5α was used as an intermediate host for the cloning and propagation of all the plasmids. The yeast expression vector pSTA1 (5.99 kb), a derivative of YIp5, was used as the integration plasmid. The 2μ plasmid, YEp*lac*112 (4.9 kb), with the *S. cerevisiae TRP1* auxotrophic marker gene, was used as the multi-copy expression vector. Both forms of plasmid possess the *S. cerevisiae* phosphoglycerate kinase I gene (*PGK1*) promoter ($PGK1_P$) and terminator ($PGK1_T$) sequences with the $MF\alpha1_S$ secretion signal, along with the ampicillin resistance gene and the sequences required for replication in both *E. coli* and *S. cerevisiae*. Plasmid pIP*LKA1* was the source of the *LKA1* gene from *L. kononenkoae* (Eksteen *et al*., 2003). *Saccharomyces cerevisiae* strain FY834 (*MATα his3 leu2 lys2 trp1 ura3 flo8*) was used as a non-flocculent strain while strain FY835 (*MATα his3 FLO8 lys2 trp1 ura3*) was used as a flocculent strain.

TABLE 1 - Strains and plasmids used in this study

Strains and Plasmids	Genotype	Reference/Source
Esherichia coli		
DH5α	*F- φ80dlacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (rK^-, mK^+) phoA supE44 λ- thi-1 gyrA96 relA1*	Ausubel *et al*. (1994)
Saccharomyces cerevisiae		
FY834	*MATα his3 leu2 lys2 trp1 ura3flo8*	Winston *et al*. (1995)
FY835	*MATα his3 flo8::FLO8 lys2 trp1 ura3*	This study
BELK1	*MATα his3 leu2 lys2 trp1 ura3::LKA1flo8*	This study
BELK1-F	*MATα his3 flo8::FLO8 lys2 trp1 ura3::LKA1*	This study
BELK100	*MATα his3 leu2 lys2 trp1 LKA1flo8*	This study
BELK100-F	*MATα his3 FLO8 lys2 trp1 LKA1*	This study
Plasmids		
YIpLac22-FLO8	Ap^R $FLO8_{PT}$ *FLO8 LEU2*	Dewald van Dyk (unpublished)
YIp5	Ap^R Tc^R *URA3*	This study
pSTA1	Ap^R $Tc^R PGK_{PT}$ *URA3*	This study
pSTA2	Ap^R $Tc^R PGK_P$-*LKA1*-PGK_T *URA3*	This study
YEpLac112	*2μm ORI* Ap^R *LacZ* PGK_P-*LKA1*-PGK_T *TRP1*	Gagiano *et al*. (2002)
YEpLK112	*2μm ORI* Ap^R *LacZ* PGK_P-*LKA1*-PGK_T *TRP1*	This study

Media and growth conditions. *Escherichia coli DH5α* was grown at 37 °C in Luria Bertani (LB) medium containing 100 μg/ml ampicillin (Ausubel *et al*., 1994). The corn starch medium used for the observation of amylolytic activity of *S. cerevisiae* transformants consisted of 20 g l^{-1} corn starch (Sigma-Aldrich, St. Louis, MO, USA), 3 g l^{-1} starch azure, 5 g l^{-1} peptone buffered with citrate buffer (pH 5.5) and 20 g l^{-1} agar. The medium used to evaluate invasive growth consisted of 20 g l^{-1} glucose as the sole carbon source, 50 μM ammonium sulfate as the sole nitrogen source, and 1.7 g l^{-1} yeast nitrogen base (YNB) without ammonium sulfate and amino acids (Difco Laboratories, MI, USA). The growth characteristics of the amylolytic strains were examined using YPRS medium containing 10 g l^{-1} yeast extract, 2 g l^{-1} peptone and 10 g l^{-1} raw corn starch. The growth pattern was monitored by sampling at 4 h intervals. For selection of yeast transformants, minimal medium (20 g l^{-1} glucose and 6.7 g l^{-1} YNB without amino acids) was supplemented with amino acids as required (50 mg l^{-1} tryptophan, 240 mg l^{-1} leucine, 50 mg l^{-1} histidine, and/or 40 mg l^{-1} uracil). The medium used for fermentation consisted of 20 g l^{-1} raw corn starch, 6.7 g l^{-1} yeast nitrogen base, 0.4 g l^{-1} Tween-80 and 0.01 g l^{-1} ergosterol (the stock solution was made up with ethanol, which contributed to 11 mM ethanol in the medium).

Construction of recombinant amylolytic plasmids and strains. The *LKA1* gene was amplified by the polymerase chain reaction (PCR) method from plasmid pIP*LKA1* with primers F-LKA1 (5′-CGGAATTCCGGAAGCTTATGGATTGCACTACAGTTA-3′) and R-LKA1 (5′-GGCTCGAGCTACATGGAGCAGATTC-3′). The PCR product was cloned into the *Hin*dIII-*Xho*I sites of pSTA1 for the construction of integration plasmid pSTA2 (7.9 kb). Subsequently, the $PGK1_P$-$MF\alpha1_S$-*LKA1*-PGK_T cassette from pSTA2 was amplified using primers F-PGK634 (5′-GCGACGTCCTTTATTTTGGCTTCACCC-3′) and R-PGK B (5′-GCGGATCCGATAAATAATAGTCTATATATACG-3′) and cloned into the 2μ plasmid YEp*lac*112 to construct the multicopy expression vector YEp*LK*112. The final constructs were verified by sequence analysis using the ABI PRISM® Big Dye™ Terminator cycle sequencing kit with an ABI PRISM™ 377 DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA). Both the coding and non-coding strands were sequenced to ensure the reliable identification of all the constructs. *Saccharomyces cerevisiae* non-flocculent strain FY834 (*MATα his3 leu2 lys2 trp1 ura3 flo8*) with a non-reverting mutation in *flo8* was transformed with a functional copy of *FLO8* to restore the flocculent phenotype (FY835). To create the amylolytic BELK1 and BELK1F recombinant strains, plasmid pSTA2 was integrated into the genomes of *S. cerevisiae* strains FY834 (non-flocculent) and FY835 (flocculent), respectively. The integration plasmids were linearised with the unique *Apa*I site within the *URA*3 sequence to ensure targeting of the *URA3* locus in the *S. cerevisiae* genome. Transformants were selected on SC medium without uracil. The transformation of FY834 and FY835 with the 2μ plasmid YEpLK112 resulted in the multicopy recombinant strains, BELK100 and BELK100F, respectively. The transformants were selected on SC medium without tryptophan. Transforming the native plasmids pSTA1 and YEplac112 into the flocculent and non-flocculent strains of *S. cerevisiae* generated the corresponding reference strains. High efficiency transformation was performed using lithium acetate method (Gietz *et al*., 1992).

Southern hybridisation. Genomic DNA was isolated from five independent colonies of each recombinant *S. cerevisiae* strain, digested with *Ns*iI, subjected to agarose gel electrophoresis and then blotted onto a nylon membrane. The blot was then probed with 1.8 kb DIG-labelled (Roche) *LKA1* gene to confirm integration of the cassette at the *URA3* locus.

Hydrolytic activity on raw starch. Recombinant strains were evaluated for their amylolytic activity on 20 g l^{-1} of raw corn starch medium consisting of 3 g l^{-1} starch azure, 6.7 g l^{-1} yeast nitrogen base with amino acids and 20 g l^{-1} agar. The cultures were incubated at 30 °C for 40 h for starch degradation to be observed.

Measurement of the flocculation ability of the yeast cells. The flocculation ability of the yeast cells was measured according to the method described by Smit *et al*. (1992). Flocculent yeast cells were dispersed by washing twice with 100 mM EDTA and twice with sterilised water. The cells were re-suspended in 3 ml of 50 mM phosphate buffer (pH 6), with and without 0.1% $CaCl_2$ added, to a final concentration equivalent to an optical density measured at 600 nm (OD_{600}) of 2. Following incubation at room temperature for 30 min, the cell suspensions were agitated for 20 s using a vortex mixer at maximum speed and then incubated for 5 min. Samples of 1 ml from the upper phase of the cell suspensions were transferred to cuvettes, and the OD_{600} was measured with and without the added $CaCl_2$. The flocculation ability (FA) was determined by means of the equation, FA=1-B/A, where A is the OD_{600} value without $CaCl_2$ and B is the OD_{600} value with $CaCl_2$. The flocculation ability value was close to 1 in cells with strong flocculation ability.

Cell surface hydrophobicity. Cell surface hydrophobicity was measured using a solvent partition assay (Powell *et al*., 2003). The assay was carried out at 4 °C. Wide-bore pipette tips were used for the flocculent cell cultures. The cells were diluted to a concentration of $5x10^6$ cells ml^{-1} in sterile distilled water, after which 10 ml aliquots of yeast samples in a serum tube were washed once and resuspended in PUM buffer (22.2 g l^{-1} K_2HPO_4, 7.26 g l^{-1} KH_2PO4, 1.8 g l^{-1} urea, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$). The absorbance of the cell suspensions (I) was measured spectrophotometrically. Aliquots of 2.4 ml yeast solution were transferred to round-bottomed test tubes. Xylene (0.2 ml) was added to each tube; the samples were vortexed for 30 s and then left to stand for 15 min. The xylene layer from each tube was removed and discarded. The aqueous layer was sampled and the absorbance was measured at 660 nm (F). The modified hydrophobicity index (MHI) was defined as I-(F/I), where a higher value indicated a hydrophobic population.

α-Amylase activity assays. Amylase activity was measured by the dinitrosalicylic acid (DNS) method (Eksteen *et al*., 2003). The reaction mixture contained 1 ml of 1% raw starch in sodium monophosphate buffer at pH 6.9 and 500 μl culture supernatant. The mixture was made up to 2 ml using distilled water. It was incubated at 50 °C for 30 min,

1 ml of DNS reagent was added, and the mixture was boiled for 15 min. After cooling, the reaction mixture was diluted with 9 ml of distilled water and the absorbance was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme causing the release of 1 mg of reducing sugars (maltose) per minute under the assay conditions.

Protein purification. A protocol based on anion exchange chromatography for the partial purification of proteins was devised using a BIO-RAD Model EP-1 Econo Pump (Bio-Rad). DEAE Sepharose™ fast flow matrix (Amersham Pharmacia, Uppsala, Sweden) was equilibrated with 50 mM of MES buffer (pH 6.0). Equal proportions of supernatant and 100 mM MES buffer (1:1 ratio) were added to the column and mixed slowly for 2 h at 4 °C using an end-to-end Labinco rotary mixer. Bound proteins were eluted using 0.2, 0.3, 0.4, 0.5, 0.7 and 1 M of NaCl. Different fractions of the sample were collected and run through a YM-3 Microcon® centrifugal filter device at 10000 *x g* for 50 min to remove the salt from the samples. Further purification and collection of proteins above 50 kDa was carried out by treatment of the eluent through a YM-50 Microcon® centrifugal filter device at 14000 *x g* for 12 min. The retentate was collected by centrifugation at 1000 *x g* for 3 min. Partially purified proteins were further analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The total protein concentration was determined using the Pierce protein reagent, with bovine serum albumin as the standard.

SDS-PAGE and Western blot. SDS-PAGE was carried out essentially as described by the supplier (Hoeffer Scientific, San Francisco, CA, USA). Proteins were resolved in a discontinuous buffer system using a Mighty Small Vertical electrophoresis unit with a 7.5% stacking gel and resolving gel. After electrophoresis, the gels were subjected to silver staining. Wide-range molecular mass standard was purchased from Sigma-Aldrich. The resolved peptides were then electrophoretically transferred onto an immobilon™ – P transfer membrane (Millipore, Billerica, MA, USA) by semidry blotting. The blotted proteins were identified immunochemically through the sequential addition of rabbit polyclonal anti-α-amylase (Abcam, Cambridge, UK), followed by donkey anti-rabbit Ig horseradish peroxidase-linked whole antibody (Amersham Pharmacia). The secondary antibody was detected using the ECL Western blotting detection system (Amersham Pharmacia).

Immunochemical quantification of proteins. The protein samples to be quantified were adsorbed overnight on sterilin (Bibby-sterilin) immunoplates at 4 °C. ELISA was performed as described by the suppliers of primary antibodies (Abcam). To prevent non-specific binding, the wells were blocked with 300 µl of 1% skim milk powder at 37 °C for 2 h. Phosphate-buffered saline at pH 7.5 was used as a washing buffer. After the plates had been washed twice, rabbit polyclonal anti-α-amylase (Abcam) was added and the samples were incubated at room temperature for 2 h. This was followed by conjugation with anti-rabbit secondary antibody linked to alkaline phosphatase. The binding of the α-amylases was detected using alkaline phosphatase and para-nitro phenol phosphate substrate. The release of para-nitro phenol was measured by absorbance at 405 nm, using a microtitre plate reader (Bio-Tek Instruments, Winooski, VT, USA). Different concentrations of α-amylase from *Bacillus amyloliquefaciens* (Sigma-Aldrich) were used to derive the ELISA standard for quantification.

Thin-layer chromatography. Thin-layer chromatography of starch hydrolysis was separated using a silica gel 60 F_{254} thin-layer chromatography plate (Merck, Whitehouse Station, NJ, USA) and a solvent system of n-propanol-ethanol-water (7:1:2). After 8 h of separation, the plate was developed with 5% sulfuric acid in ethanol spray, followed by incubation at 110 °C for approximately 5 min.

Flow-cell analyses. The flow-cell system, consisting of four channel flow cells with individual channel dimensions of 1 x 4 x 40 mm, was assembled and sterilised. The flow-cell channels were filled with 0.3% starch azure and the starch was allowed to settle in the channels. Cell counts of 2×10^7 of BELK1 and BELK1-F strains were inoculated into separate channels of the flow-cell setup in duplicate. After inoculation, the starch and cells were incubated for 1 h. The interaction of the starch and the cells was observed under the microscope. Peptone water was pumped through the channels at a constant rate of 0.2 mm/s, using a Watson Marlow 205S peristaltic pump. This rate was chosen to prevent the starch from being washed out of the channels. Observations were made under the microscope and cells washed out of the different channels were collected and enumerated.

Atomic force microscopy. A slightly modified version of the method described by Morris *et al*. (2005) was used. BELK1 and BELK1F strains were grown in YPD medium and harvested by centrifugation at 5000 *x g* for 5 min. The cells were washed with PBS (pH 8.0), and twice with distilled water. Flocculent and non-flocculent cultures with OD_{600} values of 0.2 were incubated in 1% corn starch for 30 h. Aliquot samples of 2 µl were confluently coated for observation under the atomic force microscope. The nanoscope III contact mode (Explorer) AFM (Veeco Instruments, Woodbury, NY, USA) was used to image the cells and measure the adhesive forces at different locations in the sample. Nanoprobe silicon nitride cantilevers with a spring constant of 0.06 N/m were used. Force measurements were carried out by engaging the AFM tip to scan the surfaces. The tip was moved across the surfaces in 100 nm increments, with a specified Z scan size of 300 nm at a frequency of 1 Hz. The surfaces were imaged after every force to confirm the presence of continuity in the samples. Starch samples without cells and samples of cells without starch were used for control measurements.

Scanning electron microscopy. The time frames of degradation and the pattern of hydrolysis of the starch granules by flocculent and non-flocculent strains of *S. cerevisiae* were monitored using scanning electron microscopy (SEM). Samples from the fermenter were removed at different time points and observed under a Leo435VP scanning electron microscope and they were gold-coated using the polaron SEM coating system. FY834 and FY835 were used as control strains.

Permeation of recombinant yeast cells. Recombinant strains expressing the amylolytic enzyme were perme-

abilised by treatment with isopropyl alcohol (Liu *et al*., 1998). The yeast cells were separated from the culture medium by centrifugation at 2300 *x g* for 5 min. The cell pellets were washed once with 0.85% NaCl. Wet cell weights of approximately 0.1 g were suspended in 1 ml of 40% iso-propanol and incubated at 4 °C for 10 min with shaking. The permeabilised cells were then separated by centrifugation at 5000 rpm for 5 min and washed with 0.85% NaCl.

Small-scale fermentations. Recombinant strains (pre-grown in YPD medium at 30 °C for 24 h) were aseptically inoculated into the fermentation media at a high cell density of 2 x 10^9 cell ml^{-1}. Small-scale bench fermentations were carried out with 100 ml of medium in 250 ml serum bottles plugged with rubber stoppers, and a gas outlet was secured by inserting a cannula. Fermentation was performed at 30 ?C for 90 h with agitation by magnetic stirring. Samples were taken every 12 h for analyses of the fermentation products.

HPLC analyses of sugars and fermentation products. For the HPLC analyses, the supernatant samples from the fermentation were filtered using a 0.22 μm Millipore filter. Residual starch concentrations in a 1 ml sample of undiluted fermentation broth were calculated by determining the concentration of the reducing sugars. The concentrations of reducing sugars (maltotriose and maltose), ethanol, glycerol and acetic acid were determined by HPLC using an Aminex HPX87H column (Bio-Rad, Richmond, California). The following conditions were used: mobile phase, H_2S0_4 (6 mmol ml^{-1}); flow rate, 0.8 ml min^{-1} and temperature, 65 °C, using a refractometer (Sreenath *et al*., 2001).

RESULTS

Construction of amylolytic strains with flocculent and non-flocculent phenotype

The list of plasmids and strains constructed for this study is given in Table 1. The integration of *LKA1* into the recombinant strains of *S. cerevisiae* was confirmed by Southern blotting (data not shown). Transformants carrying the multi-copy vector were confirmed by their growth on amino acid selection plates (*TRP1*). The transformation of a functional copy of *FLO8* into the strain FY834 resulted in the flocculent strain FY835. When grown on SLAD plates, the flocculent strain grew invasively into the agar plates, but was unable to use starch as a sole source of carbon. The flocculent amylolytic recombinant strain BELK1F showed adhesion and invasive phenotype behaviour on SLAD agar plates, whilst the non-flocculent amylolytic strain BELK1 did not invade agar.

Expression, purification and analysis of amylolytic activity in flocculent and non-flocculent *Saccharomyces cerevisiae*

Constitutive expression using the *PGK1* promoter and terminator and the use of the $MF\alpha1_S$ secretion signal sequences led to the expression and secretion of active biological LKA1 protein in *S. cerevisiae*, respectively. The use of the $MF\alpha1_S$ secretion signal resulted in increased secretion of biologically active protein into the extracellular media and only 10% of the enzyme was intracellular (data not shown). Neither the wild-type non-flocculent strain FY834, nor the flocculent strain FY835 exhibited α-amylase activity on raw starch substrate. All recombinant strains expressing *LKA1* showed significant amylolytic activity, which was detected by halos of degradation on corn starch plates. The α-amylase enzymes from the supernatants of the recombinants were partially purified and analysed using SDS-PAGE and Western blotting (data not shown). A clear band near 68 kDa, which represented α-amylase, was observed in the enzyme preparation of recombinant strains; the parent strain consistently did not show this band.

The amylolytic activity of the recombinant strains was analysed using raw corn starch (Table 2). Expression is essentially a linear function of the copy number; however, in this analysis the copy number did not indicate any significant increase in the enzyme activity of episomal constructs in comparison to that of the single copy integrants. Permeation of the flocculent and non-flocculent cells using iso-propanol did not illustrate any significant effect on the secretory efficiency of the cells.

TABLE 2 - Specific activity of LKA1 α-amylase from recombinant *Saccharomyces cerevisiae* strains at 48 h

S. cerevisiae strain	Specific activity (U mg^{-1} l^{-1} total protein)[a]	α-Amylase (U l^{-1})[b]
Control	11.3 ± 3.2	ND
BELK1	121.0 ± 23.5	87
BELK1F	123.8 ± 27.4	82
BELK1[P]	124.7 ± 32.1	90
BELK1F[P]	125.2 ± 31.7	85
BELK100	133.5 ± 27.9	83
BELK100F	132.6 ± 28.9	86
BELK100[P]	131.2 ± 27.7	85
BELK100F[P]	132.7 ± 29.2	87

[a] One unit of enzyme liberates 1 mg of maltose from 1% starch per minute. The presented values are the average of three independent measurements.
[b] Units of enzyme based on ELISA quantification.
[P] Permeated.

Effect of flocculation on the time window of starch hydrolysis

The wild-type strains (FY834 and FY835) and the recombinant strains (BELK1, BELK1-F, BELK100 and BELK100-F) were efficient utilisers of maltose and, when grown on maltose as the sole carbon source, the performance of both the flocculent and non-flocculent amylolytic strains was similar (data not shown). All the recombinant strains expressing *LKA1* were able to grow on raw corn starch as the sole carbon source (Fig. 1). Despite similar α-amylase levels in the supernatants, the non-flocculent strains BELK1 and BELK100 had a lag phase of 48 h before entering a logarithmic growth phase, while the flocculent strains (BELK1-F and BELK100-F) had a shorter lag period and active growth occurred after only 36 h (Fig. 1A). The qualitative analyses of the supernatants from the recombinant strains were done at time points between 12 and 90 h of growth in starch media using thin layer chromatography to observe the time frame of hydrolysis. It was noted that detectable

amounts of reducing sugars, such as maltose and maltotriose, were present in the media as early as 36 h in the flocculent amylolytic strains, but were detected in the non-flocculent strains only after 48 h (Fig. 1B and 1C). This suggested the existence of an earlier time window of hydrolysis in the flocculent phenotypes, despite similar enzyme concentrations and activities in both the flocculent and non-flocculent cells in the stipulated time period. It was also noted that maltose was readily utilised by the strains but that there was a build-up of maltotriose in the media.

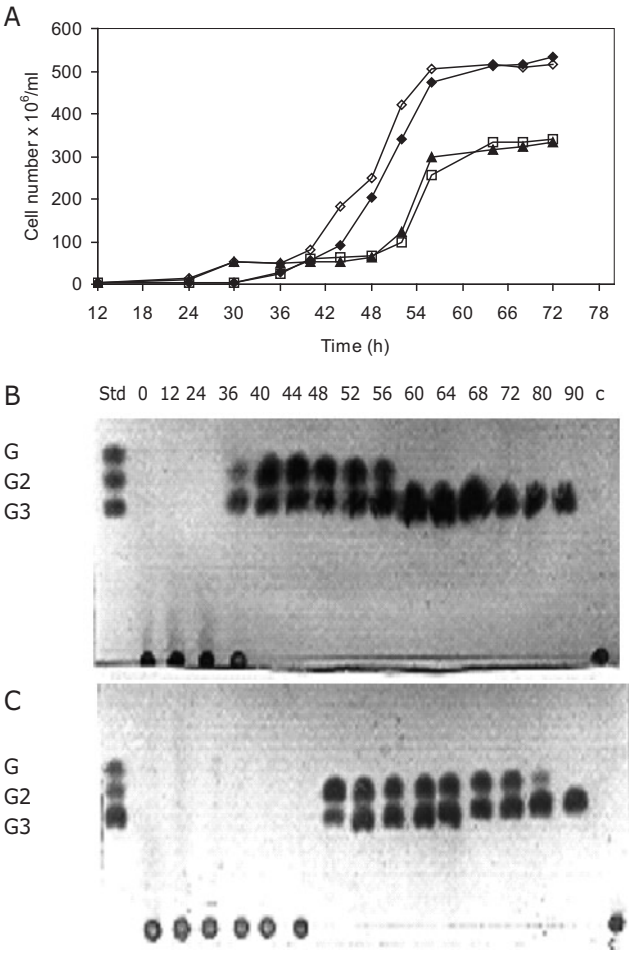

FIG. 1 - (A) Growth pattern of recombinant amylolytic strains on 2% raw corn starch: BELK1 (▲), BELK1-F (◇), BELK100 (□), BELK100-F (◆). Each value is a mean of three determinations ± standard deviation, SD < 10%. (B) Comparison of time window of active starch hydrolysis during the growth phase of flocculent and (C) non-flocculent amylolytic recombinants. G-glucose; G2-maltose; G3-maltotriose; c - non amylolytic strains (FY835 in (B) and FY834 in (C); sampling time 60 h); Std-standard mix of glucose, maltose and maltotriose.

Assessment of starch-cell interactions *in situ*

Criteria such as hydrophobicity and cell adhesion were studied to assess the properties of the flocculent cells that enabled them to shift the window of hydrolysis. A solvent partition assay showed that the flocculent cells had a higher cell surface hydrophobicity index compared to the non-flocculent strains (Fig. 2A). This property of the flocculent cells was dependent on the growth-phase, although it was observed that the flocculent cells were consistently more hydrophobic than the non-flocculent cells throughout the late log phase and the stationary phase. The invasive phenotype of flocculent strain BELK1 F is shown in Fig. 2B.

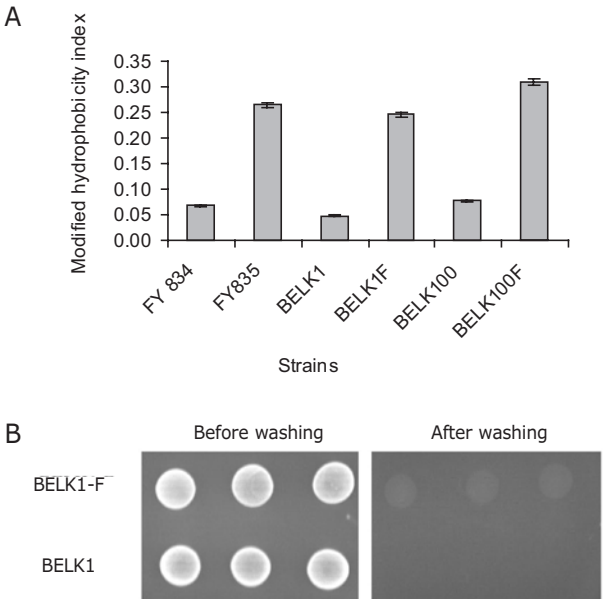

FIG. 2 - (A) Assessment of cell surface hydrophobicity of flocculent and non-flocculent *Saccharomyces cerevisiae* cultures grown in YPD for 36 h at 30 °C. Data represented are mean of two individual experiments with a standard deviation (SD) of < 10%. (B) Assessment of invasive phenotype of flocculent (BELK1F) and non-flocculent (BELK1) amylolytic *Saccharomyces cerevisiae* strains grown on SLAD agar plates for 3 days at 30 °C.

A flow-cell system was used for *in situ* observations to follow whether the higher surface hydrophobicity of the flocculent cells contributed to their interactions with starch (Table 3). Under an epifluorescence microscope it was noticed that the flocculent cells adhered to the surface crevices of the raw starch, in contrast to the non-flocculent cells, which did not exhibit such adherence properties. Representative samples of the flocculent and non-flocculent strains from the flow-cell channels revealed that, of the 10^7 cells inoculated in each flow-cell channel, only 10^3 flocculent cells were recovered in the effluent while as many as 10^5 non-flocculent cells were present in the recovered material.

TABLE 3 - Flow cell analyses of flocculent (BELK1-F) and non-flocculent (BELK1) strains in starch azure

Strains	Initial inoculum[a]	Eluted cells[a]	Retained cells[a]
BELK1F	1×10^7	1×10^2	1×10^5
BELK1	1×10^7	1×10^4	1×10^3

[a] Cells ml^{-1}

Experiments were performed in duplicate and the standard deviation was less than 10%.

Multiple force curves recorded at various locations using atomic force microscopy demonstrated single and multiple unbinding forces in about 50% of a total of 100 force measurements. A force of 120 nN ± 12 was recorded for the flocculent cell samples in the starch matrix. A lower value of 32 nN ± 7 was observed for the non-flocculent cells, suggesting little or no adhesion. This confirmed significant differences in the interactive forces between flocculent cells and non-flocculent cells incubated in starch matrix.

SEM analysis of the hydrolysis pattern

The shift in the time window of hydrolysis and differences in the hydrolytic patterns of flocculent and non-flocculent cells were observed with scanning electron microscopy (Fig. 3). In flocculent amylolytic strains, the degradation began actively at 36 h and progressed until 72 h, showing characteristic patterns of extreme pitting, shredding and liquefaction. The surface pores on the corn starch granules allowed hydrolytic attack by the enzyme and, in the later stages of degradation, a 'sieving effect' was observed in which the flocculent cells synergistically sheared the liquefied starch matrix by rapid enzymatic hydrolysis. In the non-flocculent cells, degradation was initiated after 48 h and pit formation, gritting and hydrolysis were observed. In contrast to the flocculent cells, with the non-flocculent cells, the rate and the time of 'sieving' of the starch matrix was slower and less efficient, leading to prolonged times of degradation.

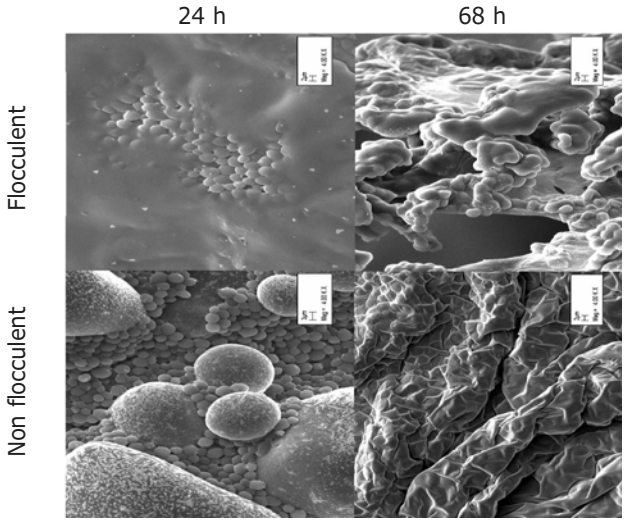

FIG. 3 - Ultra structure of starch hydrolysed by flocculent and non-flocculent amylolytic *Saccharomyces cerevisiae* at different time periods in the fermentor. All observations recorded at 4000X; scale bars are 3 μm.

Fermentation characteristics of flocculent *vs.* non-flocculent amylolytic *Saccharomyces cerevisiae*

The groups of recombinant flocculent and non-flocculent *S. cerevisiae* strains [both the permeated (BELK1P, BELK100P, BELK-FP, BELK100-FP) and the non-permeated (BELK1, BELK100, f BELK-F, BELK100-F)] were studied for their fermentative ability on raw starch in small batch-scale fermentations at the 72 h time point. By-products formed during the fermentations and the residual starch are listed in Table 4. The flocculation efficiency of the strains at the 72 h time point of the fermentation phase is also listed. The data showed that, in general, the fermentation performances of multicopy transformants were similar to those of the integrants. The data also showed that there was a modest improvement in the production of all fermentation by-products by the flocculent strains with no influence from the permeation factor. However, a significant difference was found in the residual starch concentrations with the flocculent strains showing less residual starch than the non-flocculent strains at 72 h. Further, the fermentation profiles of the integrant strains with the non-flocculent (BELK1) and flocculent (BELK1-F) phenotypes were studied in detail (Fig. 4). The non-flocculent strain consumed 76% of raw starch in the media, producing 4.61 g l^{-1} of ethanol after 90 h of fermentation, whereas the flocculent strain utilised 82% of raw starch, releasing 5.1 g l^{-1} of ethanol at 90 h. One mole of glucose (180 g) theoretically yields 2 moles of ethanol (92 g). On the assumption that all the sugar produced was converted to ethanol, it was calculated that the

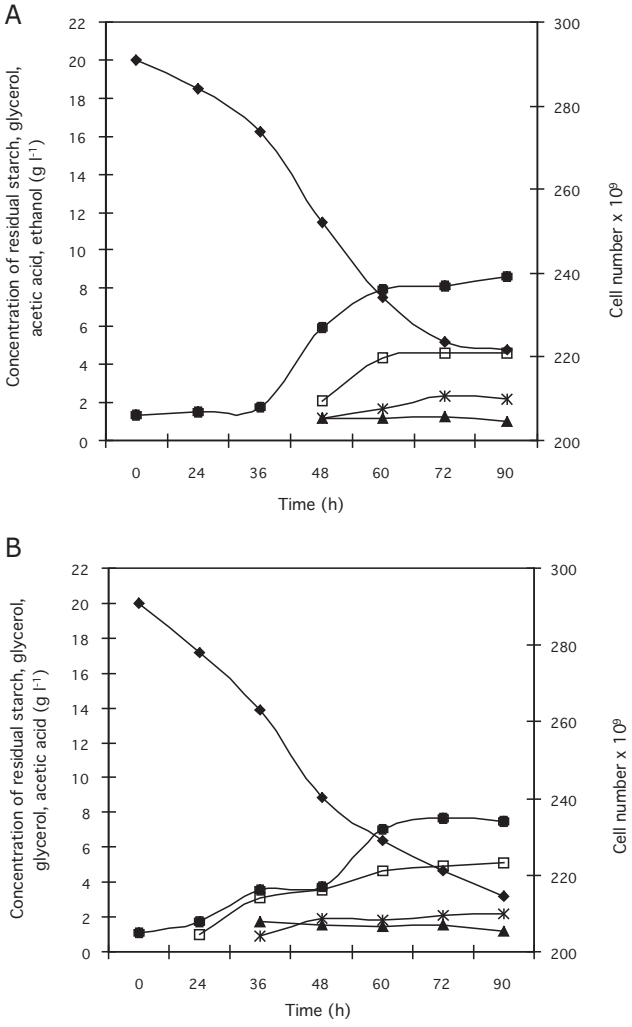

FIG. 4 - Comparison of product profiles of flocculent and non-flocculent recombinant *Saccharomyces cerevisiae* strains during small-scale batch fermentations. Cell numbers represented are the total in the fermenter. The presented values are the average of two independent measurements which varied by less than 15%. (A) Non-flocculent amylolytic strain BELK1; (B) Flocculent amylolytic strain BELK1F: (□) Ethanol; (◆) starch; (▲) acetic acid; (✳) glycerol; (●) biomass.

TABLE 4 - Comparison of starch consumption and byproducts among flocculent and non-flocculent amylolytic *Saccharomyces cerevisiae* strains at 72 h of the fermentation phase

Strain	Ethanol[a]	Glycerol[a]	Acetic acid[a]	Residual starch[a]	Flocculation ability[b]
BELK1	4.57	1.23	2.31	4.8	ND
BELK1F	4.92	1.17	2.23	3.2	53 ± 3
BELK1^{P}	4.50	1.12	2.26	5.2	ND
BELK1F^{P}	4.89	1.21	2.34	4.2	57 ± 4.5
BELK100	4.42	1.23	2.25	5.2	ND
BELK100F	4.90	1.24	2.27	4.2	59 ± 2.6
BELK100^{P}	4.62	1.17	2.24	4.8	ND
BELK100F^{P}	4.88	1.15	2.03	3.8	57 ± 2

[a] g l^{-1}; [b] %; [P] permeated; ND: not detectable.
The presented values for all byproducts are the average of three independent measurements which varied by less than 15%.

flocculent strain produced ca. 60% of theoretical yield and the non-flocculent strain resulted in 50% of ethanol yield. Detectable amounts of ethanol were formed in the media at 48 h by the non-flocculent strain, BELK1, while the flocculent strain, BELK1-F, started ethanol production as early as 36 h, without a lag in the fermentation phase. On average, the flocculent strains produced at least 0.5 g l^{-1} of ethanol more than the flocculent strains. A similar trend was also noticed in the production of other by-products, such as glycerol and acetic acid. Furthermore, an early decrease in the residual starch concentration was noticed for the flocculent strains when compared to their non-flocculent counterparts, indicating a shift in the time period of hydrolysis.

DISCUSSION

One of the main limitations of the industrially important yeast, *S. cerevisiae*, is its inability to convert relatively inexpensive polysaccharide-rich substrates, such as starch, into commercially important commodities (e.g. biofuel ethanol, low carbohydrate beer and grain whisky). The present study forms an integral part of a world-wide, long-term research effort aimed at the development of an economically viable and sustainable process for the efficient bioconversion of raw starch, along with lignocellulose, into alcohol (Lynd *et al*., 2002).

Previously, we reported that non-flocculent *S. cerevisiae* strains that express the *LKA1* and *LKA2* encoded α-amylases from *L. kononenkoae* degraded soluble starch efficiently by synergistic enzymatic activities (Steyn *et al*., 1995; Eksteen *et al*., 2003). The present study describes the effect of the flocculent phenotype on raw starch utilisation by *S. cerevisiae* strains expressing the raw starch-degrading α-amylase LKA1. Although the recombinant flocculent and non-flocculent amylolytic strains exhibited little or no difference in their α-amylase activities, a significant shift in the growth pattern and time period of raw starch hydrolysis was noticed for the flocculent strains. The data suggested that the physicochemical properties such as hydrophobicity and cell adhesion, which are associated with the surface of flocculent amylolytic strains, would have created an advantage over the non-flocculent cells in the earlier hydrolysis of starch substrate. In addition, the data suggested that flocculation by itself would have contributed towards the earlier production of ethanol because a high cell density can be maintained within the fermentor without the aid of immobilising agents (Rodriguez and Callieri, 1983). At the same time, the fact that flocculation and the invasive growth phenotype in yeast were co-regulated (Liu *et al*., 1996) implied that the improved ability of flocculent strains to act and hydrolyse starch was not only due to their increased adhesion to the substrate, but would also have been due to their ability to grow invasively into the growth substrate (Gagiano *et al*., 2002; Kobayashi *et al*., 1996; Madhani and Fink, 1998). Moreover, these adhesion properties of the flocculent cells would effectively have reduced the time interval for enzyme-substrate interactions, thus amplifying the rate of hydrolysis at an earlier time point.

Starch consists of amylose and amylopectin, which are inherently incompatible molecules, and the amylose component forms helical structures that are relatively stiff and may present contiguous hydrophobic surfaces (Immel and Lichtenthaler, 2000). During the initial enzymatic attack, the inner hydrophobic core of starch rotates when it is in contact with water, exposing the hydrophilic part of the polymer to the aqueous phase. The presence of hydrophobic surfaces in starch may interact with the hydrophobic flocculent cells, taking them into the starch matrix. The hydrolysis and degradation of the substrate subsequently occur inside the starch matrix. This mode of hydrolysis seems to be more effective because the flocculent biocatalysts do not dilute the LKA1 enzyme secreted into the surrounding medium. The encounter of non-flocculent strains with the substrate is due only to the presence of cells and starch in the same suspension, but no physicochemical interactions occur. Therefore, the enzyme secreted by the non-flocculent cells disperses into and dilutes in the liquid media, prolonging the start of effective hydrolysis and thus of fermentation. This might explain the early shift in the hydrolytic window in the flocculent cells. Scanning electron micrographs confirm this unique pattern of degradation by flocculent biocatalysts. The synergistic sieving effect of the flocculent cells on the starch matrix enables the cells to enter the fermentation phase with a shorter lag phase.

The formation of a 'yeast net' does not affect the secretion of enzyme into the media. This was confirmed by data that showed that both the permeated and non-permeated

cells had similar extracellular enzyme levels. The permeation of the cells did not affect their flocculation efficiency (Powell *et al*., 2003) or the enzyme levels. In turn, the bioconversion efficiency of the permeated cells decreased slightly because of lower metabolic rates. It has been reported that the permeability of *S. cerevisiae* increases considerably in the presence of ethanol (Mizoguchi and Hara, 1996). It therefore could be suggested that increasing ethanol concentrations could have affected cell integrity and thus the metabolic machinery of the cells.

As with various other surface display systems (Rodriguez and Callieri, 1983; Mizoguchi and Hara, 1996; Kondo *et al*., 2002; Atia *et al*., 2003; Seong *et al*., 2006), the recombinant amylolytic flocculent cells in this study used starch matrix as a surface to adhere to, resulting in earlier hydrolysis. This study has shown that the flocculent biocatalysts with suitable physicochemical properties could serve as efficient cell factories for industrial fermentations. In conclusion, this paper does not claim to advance the field appreciable closer to the goal of one-step starch processing in the sense of technological enablement; rather, its significance centres on the observation that a flocculent *S. cerevisiae* genetic background hosting heterologous amylase-encoding genes appears to boost the fermentation of starch. This might be attributed to the fact that flocculent cells have a tighter interaction with starch granules. This knowledge should be considered in the long-term strategy to develop efficient starch-degrading *S. cerevisiae* strains that could eventually be optimised for consolidated bioprocessing in which production of amylolytic enzymes, hydrolysis of starch-rich substrates, and fermentation of resulting sugars to a desired product (e.g. biofuel ethanol, low carbohydrate beer, grain whisky, etc.) occurs in a single step.

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