Appl Microbiol Biotechnol (1994) 42:28–31

ORIGINAL PAPER

K. Kavanagh · P. A. Whittaker

Application of the Melle-Boinot process to the fermentation of xylose by *Pachysolen tannophilus*

Received: 15 March 1994 / Accepted: 20 April 1994

Abstract The Melle-Boinot process (cell-recycle batch fermentation) was applied to the fermentation of xylose by two strains of the yeast *Pachysolen tannophilus* for periods of 31 days. This resulted in a significant increase in the ethanol yield and productivity from xylose by *P. tannophilus* NCYC 614 but not by strain CBS 4045. Analysis of cells of strain NCYC 614 at the end of the process revealed an increased ability to tolerate exogenous ethanol, a characteristic that was lost in the absence of selection pressure. It is postulated that the increased yield of ethanol from xylose by *P. tannophilus* NCYC 614 is a product of enhanced ethanol tolerance together with increased efficiency of the fermentation process associated with the Melle-Boinot system.

Introduction

The yeast *Pachysolen tannophilus* was the first yeast identified as being capable of ethanolic fermentation of the abundant aldopentose D-xylose (Schneider et al. 1981). An unusual feature of the fermentation of xylose by this yeast is that the process can occur under aerobic conditions. Since 1981, a variety of yeasts have been shown to be capable of the fermentation of xylose and when compared to some of these, the fermentative properties of *P. tannophilus* appear quite inferior (Du Preez et al. 1984; Delgenes et al. 1986). However, *P. tannophilus* is the only xylose-fermenting yeast identified thus far for which a conventional genetic system has been developed (James and Zahab 1983) and as such represents a useful model for the study of the genetics and biochemistry of the fermentative process (James et al. 1989; Schneider 1989)

The catabolism of xylose is a multi-step process commencing with the conversion of the aldopentose to the pentitol, xylitol, by the action of xylose reductase (Bolen et al. 1986). The xylitol is then converted to xylulose by xylitol dehydrogenase and, after phosphorylation by D-xylulose kinase to xylulose-5-phosphate, the pentose phosphate pathway may be entered (Slininger et al. 1987; Schneider et al. 1989). Aeration plays a crucial role in stimulating the fermentation of xylose by *P. tannophilus* (Du Preez et al. 1984) although thoroughly aerobic conditions lead to biomass accumulation at the expense of ethanol production (Watson et al. 1984). The level of aeration also affects the quantity of xylitol formed and excreted into the medium during xylose fermentation.

The purpose of the work described in this paper was to assess the feasibility of applying the Melle-Boinot process to the fermentation of xylose by *P. tannophilus* for periods of 31 days. The Melle-Boinot process involves harvesting the cell mass at the end of a fermentation and using this as the inoculum for the subsequent cycle (Jones et al. 1981). This process has been used to enhance ethanol productivity in wine making (Rosini 1986), in the fermentation of wood hydrolysate by *Candida shehatae* and *Pichia stipitis* (Parekh et al. 1986) and in the fermentation of xylose by immobilised cells of *P. stipitis* (Linko et al. 1986). Previous application of this process to the fermentation of 2% (w/v) xylose by *P. tannophilus* resulted in enhanced ethanol production and reduced fermentation times when cycles of 24 h duration were employed (Maleszka et al. 1981). Our objective was to establish whether it would be feasible to carry out long term fermentations (744 h) with this yeast using the Melle-Boinot process and achieve concomitant increases in ethanol productivity.

K. Kavanagh (✉)[1] · P. A. Whittaker
Department of Biology, Maynooth College, Maynooth, Co. Kildare, Ireland

Present address:
[1]National Cell and Tissue Culture Centre, Dublin City University, Glasnevin, Dublin 9, Ireland.
FAX: +353-1-704 5484

Materials and methods

Yeast strains

Two wild type strains of *P. tannophilus*, NCYC 614 and CBS 4045, were used in this work.

Media

Liquid YEPD medium consisted of 2% (w/v) D-(+)-glucose (BDH), 2%(w/v) Bacto-Peptone (Difco) and 1% (w/v) yeast extract (Oxoid). Fermentation media consisted of 6% (w/v) D-(+)-xylose and 1% yeast extract. The pH of fermentation media was adjusted to 3.0 prior to sterilisation and in all cases the individual constituents of the media were autoclaved independently at 121° C and 100 kPa for 15 min.

Fermentation conditions

All fermentations were performed at 30° C and 200 rpm in an LH Engineering orbital incubator with a working volume of 40% (i.e. 200 ml fermentation medium in a 500-ml conical flask). At the end of each fermentation the cell mass was harvested by centrifugation from the spent fermentation medium, washed once with sterile distilled water and resuspended in fresh pre-warmed medium for the commencement of the subsequent cycle. The temporal break between cycles was always less than 1 h. The first cycle in each fermentation was of 96 h while the second and subsequent cycles were 72 h in duration. The complete process consisted of ten cycles and the total fermentation time was 744 h.

Determination of ethanol tolerance

The ethanol tolerance of yeast strains was measured by inoculating a pre-determined number of cells into YEPD medium supplemented, post-autoclaving, with various concentrations of ethanol and incubating for 24 h at 30° C and 200 rpm. At the end of the incubation period the cell number for each treatment was ascertained by direct counting and the number of generations attained during the period calculated.

Determination of ethanol concentration

The ethanol concentration in fermentation sample supernatants was measured using a Pye Unicam Series 104 Chromatograph (Cambridge, UK) equipped with a flame ionisation detector. The column was packed with Parapak Q with a mesh size range of 80–100. The signals were interpreted with a Milton Roy CI-10B Integrator (Shannon, Ireland). Nitrogen (30 psi) was the carrier gas, with compressed air (40 psi) and hydrogen (30 psi) being employed for the flame.

Results

Prior to the application of the Melle-Boinot process to the fermentation of xylose by *P. tannophilus*, factors affecting conventional batch fermentation were optimised. A medium pH of 3.0 and aeration levels corresponding to a working volume of 40% (see Materials and methods) were found to be optimal. These parameters inhibited significant xylitol production. Maximum ethanol yields were attained after 90 h in conventional batch fermentation and, based on this data and the results of pilot studies, it was decided that the first cycle of the Melle-Boinot process should be 96 h long and that each subsequent cycle should be of 72 h duration.

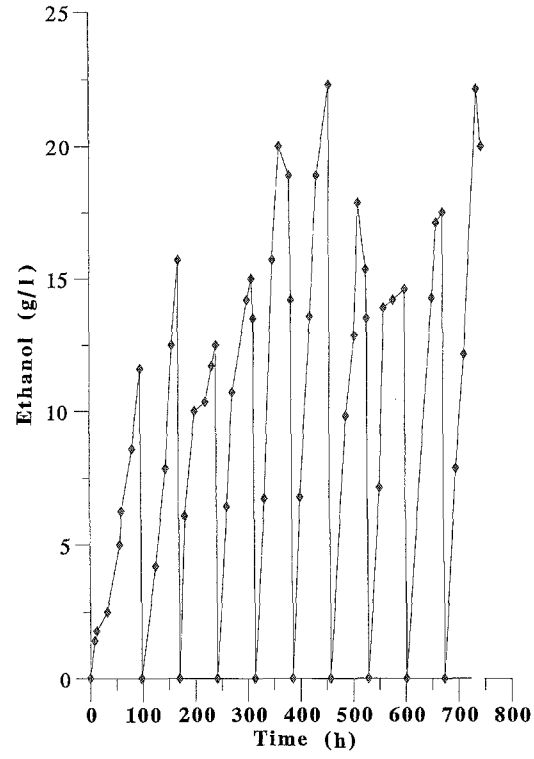

Fig. 1 Application of the Melle-Boinot process to the fermentation of 6% (w/v) xylose by *Pachysolen tannophilus* NCYC 614. The fermentation conditions are described in Materials and methods. Each point is the mean of three determinations In all cases the standard error was less than 5%

The first cycle of each fermentation was initiated using an inoculum of 1.5×10^6 cells/ml. Over the course of the fermentation process *P. tannophilus* NCYC 614 displayed a significantly greater cell density than strain CBS 4045. In the second and subsequent cycles strain NCYC 614 had a mean cell density of 2.1×10^9 whereas CBS 4045 had an average density of 1×10^9 (data not shown).

The ethanol yields in the *P. tannophilus* NCYC 614 fermentation increased significantly over the course of the process (Fig. 1). At the end of the first cycle a level of 11.6 g/l had been attained but by cycle 6 a peak ethanol concentration of 22.3 g/l had been achieved. In contrast with this, the ethanol yields from xylose by strain CBS 4045 appeared relatively constant over the course of the fermentation (Fig 2). The rate of ethanol production (g/l per hour) from xylose by strain NCYC 614 (Table 1) is superior in the second and subsequent cycles to that evident in the first cycle. The data for strain CBS 4045 demonstrate that the productivity remained relatively constant throughout the process.

At the end of the Melle-Boinot process, cells of both strains were grown in YEPD medium supplemented

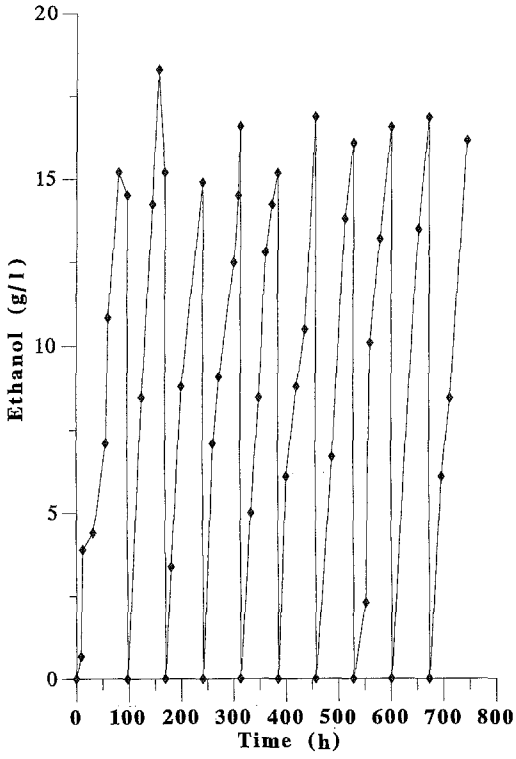

Fig. 2 Application of the Melle-Boinot process to the fermentation of xylose by *P tannophilus* CBS 4045. The fermentation conditions are described in Materials and methods. Each point is the mean of three determinations. In all cases the standard error was less than 5%

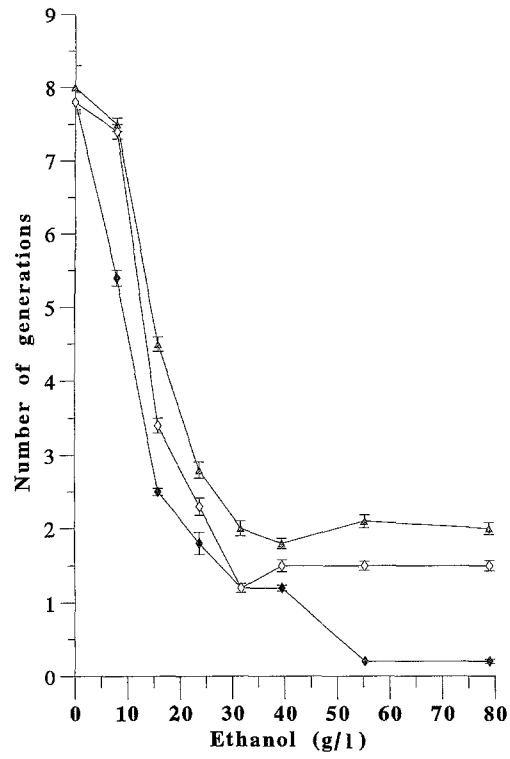

Fig. 3 Ethanol tolerances of strains of *P tannophilus* NCYC 614, measured as described in Materials and methods. Control cells (◆) had not been recycled, 2% XYL R (▲) had been recycled on 2% xylose for 31 days and 6% XYL R (◇) had been recycled, as described in the text, on 6% xylose. All values are the mean of three determinations +/- the standard error

Table 1 Rates of production of ethanol from xylose by *Pachysolen tannophilus* NCYC 614 and CBS 4045

Cycle	Rate of ethanol production (g/l per hour)	
	NCYC 614	CBS 4045
1	0.11	0.20
2	0.26	0.22
3	0.17	0.19
4	0.22	0.18
5	0.42	0.19
6	0.40	0.19
7	0.32	0.23
8	0.28	0.29
9	0.24	0.23
10	0.35	0.22

with various levels of ethanol to establish whether the continued recycling had affected their ability to tolerate ethanol. It has been shown previously that recycling could affect a number of cellular functions (Parekh et al. 1986; Talbot and Wayman 1989). The number of generations attained after incubation at 30° C and 200 rpm for 24 h by strain NCYC 614 was calculated and the results are displayed in Fig. 3. The data indicate that the cells obtained from cultures that had been repeatedly recycled during the course of the Melle-Boinot process are more resistant to ethanol levels in excess of 40 g/l than the reference strain. Cells obtained after recycling in 2% (w/v) xylose (fermentation results not shown) were consistently more tolerant to ethanol than either the 6%-xylose-recycled strain or the reference strain. The enhanced ethanol tolerance of the recycled strains was not evident after sub-culturing in ethanol-free YEPD, suggesting that the acquisition of tolerance was not the product of a genetic alteration but rather of a phenotypic change, which was lost in the absence of selection pressure.

Discussion

The data presented here indicate that the application of the Melle-Boinot process to the fermentation of xylose by *P. tannophilus* resulted in the attainment of ethanol concentrations that were equivalent to (strain CBS 4045) or significantly superior (strain NCYC 614) to those achieved in the initial batch fermentations. In addition it has been demonstrated that the fermentation times were reduced in the second and subsequent cycles of the process. Both strains of *P. tannophilus* were still capable of producing ethanol at the end of the procedure, demonstrating that this yeast is amenable to long-term fermentations.

The enhanced ethanol productivity and yield displayed by *P. tannophilus* NCYC 614 may have been a result of the recycling of the biomass at the end of each cycle, obviating the requirement for cell proliferation and enzyme synthesis. Such an improvement may have been possible since xylose fermentation by this yeast is a relatively inefficient process (Slininger et al. 1987) and so there is the potential for fermentation-system-based optimisation.

Examination of cells of strain NCYC 614 that had been recycled on xylose revealed strains possessing higher ethanol tolerances than that displayed by the control. This was not apparent in cells of strain CBS 4045 that had been similarly recycled and it was postulated that the enhanced ethanol tolerance of NCYC 614 was a product of the increased efficiency of the fermentation process whereas application of the Melle-Boinot process to the fermentation of xylose by CBS 4045 resulted in no such increase in ethanol yield. The enhanced ethanol tolerance of cells of NCYC 614 was most obvious at ethanol levels exceeding 40 g/l but was lost upon sub-culturing in ethanol-free YEPD. The acquisition of enhanced ethanol tolerance may have been the result of a stress situation, the retention of which was unnecessary for normal cell growth in the absence of the stress. The continual recycling of cells in fermentation media during the Melle-Boinot process may have constituted such a stress and necessitated the acquisition of an elevated ethanol tolerance, together with other unobserved traits, in an effort to protect the cells from ethanol-induced damage. Recycling strains of *C. shehatae* and *P. stipitis* in wood hydrolysate medium resulted in an increase in ploidy which was thought to be a result of the stress imposed by the fermentation regime (Talbot and Wayman 1989). Alternatively, during the recycling process, a sub-population of cells displaying elevated ethanol tolerance may have been selected for. The acquisition by *C. shehatae* and *P. stipitis* of enhanced ethanol productivity from various substrates was attributed to the selection for an adapted strain over the period of the fermentation (Parekh et al. 1986).

The data presented in this paper extend previous observations (Maleszka et al. 1981) and demonstrate that *P. tannophilus* is amenable to long-term fermentations of xylose using the Melle-Boinot process. Application of this procedure to the fermentation of xylose by *P. tannophilus* resulted in shorter fermentation times and ethanol levels comparable to or significantly greater than those achieved using conventional batch fermentation. Recycled cells of one strain (NCYC 614) also displayed enhanced ethanol tolerance.

Acknowledgement The authors are grateful for financial support provided by EOLAS – the Irish Science and Technology Agency.

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