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STABILITY AND EXPRESSION OF A CLONED α-GLUCOSIDASE GENE IN *ZYMOMONAS MOBILIS* GROWN IN BATCH AND CONTINUOUS CULTURE

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SUMMARY

Strains of *Zymomonas mobilis* containing an α-glucosidase gene cloned from *Bacillus brevis* strain 27-7 (NRRL B-4389) on the plasmid pNSW358 showed varying degrees of stability in batch culture under non-selective conditions. After 45 generations of growth in continuous culture, pNSW358 was stable in *Z.mobilis* strain ZM6100 and the specific activity of α-glucosidase in these cells was 2.7 nmol/min/mg protein. Lysed cell extracts confirmed the activity of the α-glucosidase enzyme in ZM6100(pNSW358) with 21 g/l ethanol in 50 h (82% theoretical conversion of maltose to ethanol). ZM6100(pNSW358) whole cells showed a very slow conversion rate on maltose as a sole carbon source with only 5.3 g/l ethanol after 30 days on 100 g/l maltose medium.

INTRODUCTION

Zymomonas mobilis is a Gram-negative bacterium capable of producing ethanol at a rate three- to four-fold faster and at a higher final yield than traditionally used yeasts (Rogers *et al.*, 1982). This organism, however, has a limited substrate range and can only utilise glucose, fructose and sucrose (Swings and DeLey, 1977). *Z.mobilis* has been nominated as an ideal ethanol producer (Esser and Karsch, 1984) if the problem of its narrow substrate range can be overcome.

The enzyme α-glucosidase is distributed widely in microorganisms and hydrolyses α-1,4 and/or the α-1,6 glucosidic linkages in short chain saccharides. Alpha-glucosidases exhibit a range of substrate specificities and those from *B.brevis* show a high activity towards maltose (Kelly and Fogarty, 1983). As the linear component of starch yields almost 100% maltose with enzymatic treatment (Brautlecht, 1953), a *Zymomonas* strain producing α-glucosidase would be the first step towards the fermentation of starch-based substrates.

We have successfully cloned an α-glucosidase gene from the Gram-positive bacterium *B.brevis* into *Z.mobilis* (Strzelecki *et al.*, 1993). In this study we report the maintenance and expression of the cloned α-glucosidase gene in *Z.mobilis* grown without selection in both batch and continuous culture. We have also investigated the efficiency of conversion of maltose to ethanol by ZM6100(pNSW358).

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The *Z.mobilis* strain used was ZM6100, which is a methionine-requiring derivative of ZM6 (Skotnicki *et al.*, 1983) containing only the two smallest of the three native plasmids of ZM6 (Browne *et al.*, 1984). The *E.coli* strain used was HB101 (Boyer and Roulland-Dussoix, 1969). Plasmids used in this study were pRK404, a 10.6kb, IncP-1 Tc^R Mob^+ plasmid (Ditta *et al.*, 1985); pRK2013, a 48kb, IncP-1 Km^R Tra^+ mobilising plasmid (Figurski and Helinski, 1979); and recombinant plasmids pNSW353, pNSW355, and pNSW358 which were isolated from three individual colonies following transformation. These plasmids show homology with the *B.brevis* α-glucosidase gene and α-glucosidase activity was confirmed by enzyme assays. These recombinant plasmids contain the α-glucosidase gene on a 4.5 kb fragment in the vector pRK404 (Strzelecki *et al.*, 1993).

Media and Growth Conditions

E.coli strains were grown at 37°C in Luria broth (LB, Miller, 1972) or on KW plates which contained per litre 4 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.5 g yeast extract, 2 ml 1.64% Fe.EDTA, 5g carboxymethyl cellulose (CMC), 15g agar (after Kim and Wimpenny, 1981). *Z.mobilis* was grown in liquid RM (Goodman *et al.*, 1982) containing either glucose and/or maltose, autoclaved separately, as the sole carbon source. Minimal media, BM, (Goodman *et al.*, 1982) was supplemented with 50 μg of methionine/ml. *Z.mobilis* cultures were incubated statically at 30°C in tightly capped 10 ml centrifuge tubes or 25 ml McCartney bottles. If required antibiotics were added to the medium as specified.

Cultivation Conditions

ZM6100(pNSW353), ZM6100(pNSW355) and ZM6100 (pNSW358) were grown in batch culture at 30°C in 10% glucose RM broth with or without tetracycline, 20 μg/μl (Tc_{20}). Continuous culture experiments involving ZM6100(pNSW358) on the same medium were conducted in a gently agitated 1 litre culture vessel controlled at 30°C, pH 5.0 with no aeration. The initial inoculum was obtained from an overnight batch culture grown in 10 ml of RM at 30°C. This culture was then added to 90 ml of continuous culture medium and after incubation at 30°C for a further 12-18 hours it was used to inoculate 900 ml of the same medium in the culture vessel. After 12-18 hours of batch culture conditions a continuous flow of medium was started at a dilution rate of $0.15h^{-1}$.

Monitoring Antibiotic Resistance and a-glucosidase Activity

Cultures growing under batch conditions were subcultured every 24 hours (1:100 dilution). Before subculturing, appropriate serial dilutions were plated onto RM plates. After three days incubation at 30°C, plates containing 200-500 colonies were replica plated onto $RMTc_{20}$ and $BMmetTc_{20}$ plates. After incubation for a further three days, colonies on $BMmetTc_{20}$ plates were tested for α-glucosidase activity by assaying with the α-MUG overlay method (Strzelecki *et al*, 1993). This procedure enabled monitoring of the maintenance of the vector pRK404 and of the 4.5 kb fragment containing the α-glucosidase gene in ZM6100 transconjugants. Samples from the continuous culture experiments were tested similarly at 24 hour intervals.

Preparation of Lysed Cells

Cells from 10 ml overnight cultures of ZM6100(pNSW358) and ZM6100 (pRK404), grown at 30°C in $RMTc_{20}$ media, were pelleted by centrifugation, washed and recentrifuged to remove any residual media. The cell pellet was lysed with glass beads and resuspended in 1 ml of phosphate buffer pH 6.5. The lysed cell suspension was then added to various media. Samples were taken at various time intervals and the amount of ethanol produced was analysed.

Ethanol Analysis

Ethanol concentrations were determined using a Technicon Autoanalyser Model 27.

Plate Assay Using 4-methylumbelliferyl-a-D-Glucoside (a-MUG)

This method was adapted for α-glucosidase activity estimations from a method developed for β-glucosidase estimations, using β-MUG (J.M. Watson unpublished results). Colonies were grown on minimal medium for three days before being tested. The plates were overlayed with 0.7% agarose dissolved in phosphate buffer pH 7.0 containing 0.4 mg 4-methylumbelliferyl-1,4-α-glucopyranoside (α-MUG)/ml (Sigma, USA) which was first dissolved in N,N-dimethylformamide (Ajax). Once the overlay had set the plates were incubated at 37°C and examined under UV light. Any colony which possesses α-glucosidase activity hydrolyses the substrate α-MUG to glucose and methylumbelliferone (MU), which produces a fluorescence under UV light.

α-Glucosidase Assay

Alpha-glucosidase activity was estimated by measuring the p-nitrophenol (pNG) released from p-nitrophenyl-α-D-glucoside (pNPG)(Sigma, USA). Strains to be tested were grown overnight and the cultures were then centrifuged, washed and recentrifuged. Cells were lysed by the addition of glass beads, followed by the addition of 1 ml of phosphate buffer (0.5 M KH_2PO_4.NaOH) pH 6.5. One ml of lysate (or a dilution thereof) was added to 1 ml of phosphate buffer pH 6.5 and 1 ml of 0.1% pNPG was added. The assay was carried out at 50°C in a water bath without shaking. The reaction was stopped by the addition of 2 ml of 1 M Na_2CO_3. Standards consisted of various known concentrations of pNP in buffer, incubated at 50°C and stopped by the addition of Na_2CO_3. Samples were centrifuged to remove any cell debris and were read at an absorbance of 400 nm on a Pye Unicam SP6-550 spectro-

photometer. One unit of enzyme activity was defined as the amount of enzyme that liberated one nmol of pNP per minute. Limits of sensitivity were 0.01 mU/ml of culture for cell-associated fractions. The specific activity was defined as enzyme activity per mg protein.

Determination of Protein

Standard curves of absorption at 600 nm versus protein concentration, using the Folin-Ciocalteu reagent and the method reported by Lowry *et al.* (1951) were used.

Colony Hybridisations

After growing colonies on well dried plates, they were placed at 4°C for 15-30 minutes. Marked nitrocellulose discs were then placed over the colonies and left in contact for 5 minutes. The discs were removed and placed on Whatman 3 MM paper moistened with 0.5 M NaOH and 0.5 M NaCl, for 10 minutes, then transferred to 3 MM paper moistened with 0.5 M Tris, pH 7.2 and 2 M NaCl, for 10 minutes. The discs were then transferred to 3 MM paper moistened with 2 x SSC, for at least 10 minutes. The discs were air dried and baked at 80°C for 2 hours. A 4.5 kb fragment, containing the α-glucosidase gene was labelled by nick translation (Rigby *et al.*, 1977) and was used as a probe.

RESULTS

The stabilities of pNSW353, pNSW355 and pNSW358, all containing the α-glucosidase gene in the vector pRK404 were investigated in ZM6100 under batch culture conditions with no selective pressure. The results presented in Table 1 show that Tc resistance was maintained at a high level, but the maintenance of a-glucosidase activity was progressively lost in some strains. ZM6100(pNSW358) which was the most stable in batch culture was examined in continuous culture (Table 2). After 45 generations of growth in continuous culture without selection, 100% of ZM6100(pNSW358) colonies were resistant to Tc, and at least 98% showed α-glucosidase activity when screened using the α-MUG overlay method. The specific activity of α-glucosidase in ZM6100(pNSW358) at the end of the fermentation run was 2.7 nmol/min/mg protein. This level of α-glucosidase activity was slightly higher than that obtained for ZM6100(pNSW358) grown in batch culture, which was 2.3 nmol/min/mg protein.

Table 1: Stability of pNSW353, pNSW355 and pNSW358 in ZM6100 transconjugants in non-selective batch culture

Strain	Number of Generations	% Maintenance	
		Tc^R*	α-Glucosidase activity*
ZM6100(pNSW353)	5	100	98
	15	98	90
	25	99	86
	50	98	72
ZM6100(pNSW355)	5	98	90
	15	98	73
	25	98	60
	50	98	40
ZM6100(pNSW358)	5	100	100
	15	100	98
	25	98	98
	50	100	96

Table 2: Stability of pNSW358 in ZM6100 in non-selective continuous culture

No. of Generations	% Maintenance	
	Tc^R*	α-Glucosidase activity*
3	100	99
6	100	99
25	100	99
35	100	98
45	100	98

(* 400-500 colonies tested)

The activity of the α-glucosidase enzyme in ZM6100(pNSW358) was investigated further to determine if this strain could cleave maltose to glucose. This was achieved by lysing overnight cultures of ZM6100(pNSW358) and ZM6100(pRK404) and adding the lysed cell suspension to 5% maltose or 5% glucose, or a mixture of 5% maltose plus 5% glucose media. The amount of ethanol produced was then analysed. The results presented in Table 3 show that the α-glucosidase enzyme in *Zymomonas mobilis* was functional and that ethanol was produced from maltose. The lysed ZM6100(pNSW358) cells produced 19.4 g/l ethanol from 5% glucose (80% theoretical conversion) after 50 hours. The lysed ZM6100(pNSW358) cells which were incubated in 5% maltose produced 21 g/l ethanol (82% theoretical conversion) after 50 hours and the lysed ZM6100(pNSW358) cells incubated with both 5% glucose and 5% maltose produced 32 g/l ethanol (64% theoretical conversion) after 50 hours. As expected, the lysed ZM6100(pRK404) cells failed to produce any ethanol from 5% maltose. The production of ethanol from 5% glucose plus 5% maltose with this strain showed that only 22.2 g/l ethanol was produced after 50 hours, indicating that no maltose was hydrolysed.

Table 3: Ethanol production by lysed cells of recombinant ZM6100

Medium	Time (h)	Ethanol produced (g/l) ZM6100(pNSW358)	ZM6100(pRK404)
5% Glucose RM	0.08	2	2.2
	0.5	2.3	2.5
	5.0	8	8.2
	22.5	18	19
	50	19.4	22
5% Maltose RM	0.08	2	<0.1
	0.5	2.2	<0.1
	5.0	5	<0.1
	22.5	20	<0.1
	50	21	<0.1
5% Glucose + 5% Maltose RM	0.08	2.7	2
	0.5	3.4	2.3
	5.0	5.7	6.4
	22.4	24	19.3
	50	32	22.2

As the α-glucosidase enzyme was functional in ZM6100(pNSW358), the strain was tested for growth on maltose and production of ethanol by whole cells. After growing overnight in 10% glucose RM, the culture was washed twice to remove residual medium and the cell pellet was resuspended in liquid RM containing 10% maltose as the sole carbon source. The concentration of the cells in the maltose medium was about 7×10^9 cells/ml. The culture was incubated at 30°C and assayed for ethanol production after 5, 8, 15 and 30 days. The amount of ethanol produced was 0.2, 1.8, 3.5 and 5.3 g/l respectively. Although the amount of ethanol produced after 30 days was low (10.4% conversion), the results show that whole cells of ZM6100(pNSW358) were capable of producing ethanol from maltose. The strain ZM6100(pRK404) was also grown up and inoculated into RM medium (containing 10% maltose) in a similar manner. No ethanol production was detected after 30 days.

An overnight culture of ZM6100(pNSW358) was washed twice, concentrated and then plated onto RM plates containing 20 g/l maltose. ZM6100(pNSW358) was unable to form colonies on the maltose plates. A sample was also streaked onto $RMTc_{20}$ and $BMmetTc_{20}$ plates. Colonies formed on these plates. The colonies on $BMmetTc_{20}$ plates were tested for α-glucosidase activity using the α-MUG overlay method and all colonies tested (approximately 800) showed fluorescence under UV light, thus indicating that a-glucosidase was active.

To confirm that the α-glucosidase gene was the same as that originally cloned from *B.brevis,* 13 colonies obtained after 45 generations of growth of ZM6100(pNSW358) in continuous culture were patched onto $RMTc_{20}$ plates. As a control, 13 RR1(pNSW350) colonies were patched onto $RMTc_{20}$ plates. RR1(pNSW350) was the original isolate which possessed the *B.brevis* α-glucosidase gene in the vector pUC118 (Strzelecki *et al.*, 1993). Other controls included *E.coli* HB101, *B.brevis*, ZM6100 and ZM6100(pRK404) which were

patched onto RM plates. DNA hybridisation studies were carried out using the colony hybridisation technique. The original 4.5 kb fragment, containing the α-glucosidase gene was radioactively labelled by nick translation (Rigby *at al.*, 1977) and used as a probe. Results established that the fragment containing the α-glucosidase gene showed strong homology to RR1(pNSW350) colonies from which the fragment was isolated. Homology was also seen between the 4.5 kb fragment and the ZM6100(pNSW358) colonies. ZM6100 (pRK404), ZM6100 and HB101 failed to show any homology with the 4.5 kb α-glucosidase containing fragment. *B.brevis* did show strong homology to the 4.5 kb fragment as expected.

DISCUSSION

Three strains of *Z.mobilis* containing a *B.brevis* α-glucosidase gene cloned into the vector pRK404 showed a high level of maintenance of Tc resistance (carried by pRK404), while α-glucosidase activity was progressively lost in some strains under non-selective batch culture conditions. The reason for this is unknown. One strain, ZM6100 (pNSW358) which was the most stable in batch culture was tested in continuous culture without selective pressure for 45 generations. It was found that the vector and α-glucosidase activity were stably maintained by ZM6100(pNSW358) cells and the specific α-glucosidase activity produced by ZM6100 (pNSW358) was 2.7 nmol/min/mg protein.

Although the cloning of various genes into *Z.mobilis* has been reported by several groups: β-galactosidase (Carey *et al.*, 1983; Goodman *et al.*, 1984), *gal* operon (Goodman, 1985), cellulase genes (Misawa *et al.*, 1988), xylose catabolic genes (Liu *et al.*, 1988), β-glucosidase (Misawa and Nakamura, 1989; Su *et al.*, 1989) and α-amylase (Brestic-Goachet *et al.*, 1990), there have been only a few reports dealing with the stability of the cloned genes in these strains in batch or continuous culture (Strzelecki *et al.*, 1986). It appears from various studies that the fate of a plasmid-bearing strain in continuous culture depends on many factors which include the nature of the plasmid, the host, the limiting nutrient, the dilution rate and the temperature. The cloning vector used here was the IncP-1 plasmid, pRK404. Members of the IncP-1 group of broad-host-range plasmids have been successfully conjugated into *Z.mobilis* strains (Dally *et al.*, 1982; Stokes *et al.*,1982; Carey *et al.*, 1983; Eveleigh *et al.*, 1983; Skotnicki *et al.*, 1983; Goodman, 1985). The plasmid pRK290, a derivative of RK2 (Ditta *et al.*, 1980), can be mobilised into a number of *Z.mobilis* strains but at a low frequency (Dally *et al.*, 1982; Carey *et al.*, 1983). The plasmid pRK404 is a derivative of pRK290 (Ditta *et al.*, 1985) which has been successfully mobilised into the following Gram-negative bacteria; *Rhizobium meliloti, Pseudomonas putida, Rhodopseudomonas sphaeroides, Acinetobacter calcoaceticus, Caulobacter crecentus* (Ditta *et al.*, 1985) and also to *Z.mobilis* ZM6100. While the broad-host-range replication and transfer capability of pRK404 are comparable to pRK290 (Thomas and Smith, 1986), Ditta *et al.*, (1985) have found that pRK404 was quite unstable in the absence of selection. This contrasts with the relative stability of pRK290 in most hosts (Thomas and Smith, 1986) and also with the findings reported in this study.

The activity of the cloned α-glucosidase enzyme on maltose was confirmed by incubating cell extracts of ZM6100(pNSW358) with glucose, maltose and a mixture of glucose plus maltose. Ethanol production was detected in all samples indicating that the α-glucosidase was hydrolysing the maltose to glucose and that normal *Z.mobilis* enzymes were converting the glucose to ethanol. No ethanol was produced from maltose by ZM6100 (pRK404). Ethanol production from cells incubated with glucose plus maltose showed that ZM6100(pRK404) produced 22.2 g/l ethanol, indicating that only the glucose was converted to ethanol, while lysed cells of ZM6100(pNSW358) produced 32 g/l ethanol in 50 hours which showed that some of the maltose was converted to glucose. It is possible that the glucose may have had an inhibitory effect on the expression of the gene encoding α-glucosidase. Here the glucose would be utilised by the ZM6100(pNSW358) cells in preference to maltose. Further studies are needed to determine how long it would take ZM6100(pNSW358) lysed cells to fully utilise the maltose and if catabolic repression by glucose is affecting the rate of hydrolysis of maltose and the subsequent production of ethanol.

Z.mobilis has been found to produce ethanol from maltose (this study), lactose (Goodman *et al.*, 1984), galactose (Goodman, 1985) and cellobiose (Su *et al.*, 1989) using intact cells, but the process is too slow to be commercially viable. Although the level of ethanol production was lower than that found with *Z.mobilis* strains containing the *lac* or the *lac* and *gal* operons (Goodman, 1985), it demonstrates that *Z.mobilis* cells containing the α-glucosidase

gene from *B.brevis* are able to produce ethanol from maltose as the sole carbon source. Although ethanol production from maltose was observed in liquid medium, ZM6100 (pNSW358) failed to form colonies on plates containing maltose. The reason for this may have been due to a permeability problem, with maltose being able to enter the cells only slowly by passive diffusion as it appears likely that *Z.mobilis* lacks the necessary transport system for maltose uptake.

This is the first reported case of the stable expression of α-glucosidase in *Z.mobilis* grown in continuous culture and of the production of ethanol from maltose by ZM6100 (pNSW358). Further work to achieve fast, efficient conversion of maltose to ethanol by *Z.mobilis* (α-glucosidase$^+$) should involve the development of strains in which the enzyme activity as well as maltose permeability are increased.

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