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Simultaneous ethanol and bacterial ice nuclei production from sugar beet molasses by a *Zymomonas mobilis* CP4 mutant expressing the *inaZ* gene of *Pseudomonas syringae* in continuous culture

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Aim: The aim of this work was to construct a *Zymomonas mobilis* mutant capable of simultaneous ethanol and ice nuclei production from agricultural by-product such as sugar beet molasses, in steady-state continuous culture.

Methods and Results: A sucrose-hypertolerant mutant of *Z. mobilis* strain CP4, named suc_{40}, capable of growing on 40% (w/v) sucrose medium was isolated following N-methyl-N′-nitro-N-nitrosoguanidine treatment. Plasmid pDS3154 carrying the *inaZ* gene of *Pseudomonas syringae* was conjugally transferred and expressed in suc_{40}. The potential for simultaneous ethanol and bacterial ice nuclei production was assessed in steady-state continuous cultures over a range of dilution rates from 0·04 to 0·13 h^{-1}. In addition, the fatty acid and phospholipid profile of the three strains was also investigated. Ethanol production up to 43 g l^{-1} was achieved at dilution rates below 0·10 h^{-1} in sugar beet molasses. Ice nucleation activity gradually increased with increasing dilution rate and the greatest activity, −3·4 log (ice nuclei per cell), was observed at the highest dilution rate (0·13 h^{-1}). Both mutant strains displayed a different fatty acid and phospholipid profile compared with the wild-type strain.

Conclusions: The ability of the mutant and recombinant plasmid-containing strains to grow on high sugar concentrations and in high osmotic pressure environments (molasses) can be attributed to their phospholipid and fatty acid contents.

Significance and Impact of the Study: Taking into account that sugar beet molasses is a low cost agricultural by-product, the simultaneous ethanol and bacterial ice nucleation production achieved under the studied conditions is considered very promising for industrial applications.

INTRODUCTION

Zymomonas mobilis, a Gram-negative bacterium, ferments glucose, fructose and sucrose exclusively via the Entner-Doudoroff pathway, yielding 1 mole ATP per mole sugar, with 96% efficiency (Buchholz *et al*. 1987). It can produce up to 12% (w/v) ethanol, at a rate faster than yeast (Rogers *et al*. 1980; Buchholz *et al*. 1987). The best performing *Z. mobilis* strain known so far is CP4 which can convert high concentrations of glucose to ethanol (Viikari 1984; Osman and Ingram 1985; Osman *et al*. 1987; Barnell *et al*. 1990; Haejung *et al*. 1991). Its physiology has been studied extensively (Bringer-Meyer and Sahm 1988) as well its ability to produce ethanol from renewable agricul-

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tural resources, such as cellulose hydrolysate (Kademi and Baratti 1996), sugar cane molasses (Doelle *et al*. 1991), sugar beet extracts (Kolios *et al*. 1989), sugar beet molasses (Park and Baratti 1991a,b) or starch (Weuster-Botz *et al*. 1993).

Despite its ability to grow uninhibited in complex media, poor ethanol production has been observed on some industrial substrates, including molasses and cellulose hydrolysate (Bringer-Meyer and Sahm 1988; Doelle *et al*. 1990; Struch *et al*. 1991). A number of different *Z. mobilis* strains have been developed in an effort to produce high value products such as fructose (Doelle and Doelle 1991), sorbitol (Rehr *et al*. 1991), β-carotene (Misawa *et al*. 1991), L-alanine (Uhlenbusch *et al*. 1991) and bacterial ice nuclei (Varsaki *et al*. 1998).

Ice nucleation active bacteria (INA) able to catalyse ice formation at temperatures below $-1{\cdot}5\,^{\circ}C$ were first reported three decades ago (reviewed by Upper and Vali 1995). Since then, extensive work has been undertaken to identify the bacterial ice nucleator and to characterize its components (Fall and Wolber 1995). Bacterial *ina* genes confer an ice^{+} phenotype when cloned and expressed in Gram-negative bacteria such as *Escherichia coli* (Orser *et al*. 1983), *Agrobacterium tumefaciens* (Lindgren *et al*. 1989), Z. mobilis (Drainas *et al*. 1995) and moderate halophiles (Arvanitis *et al*. 1995). The aim of this work was to construct a *Z. mobilis* mutant capable of simultaneous ethanol and ice nuclei production, from agricultural precursors such as sugar beet molasses, in steady-state continuous culture.

MATERIALS AND METHODS

Strains, plasmids and culture conditions

Escherichia coli strains were grown in Luria-Bertani (LB) liquid or solid media. *Zymomonas mobilis* CP4 strains were grown on liquid or solid complete medium (CM) at 24 °C (Afendra and Drainas 1987). A chemically defined medium containing sucrose instead of glucose as its sole carbon source was named complete sucrose medium (CSM) (Jones and Doelle 1991). Similarly, a 20% (w/v) solution of sugar beet molasses in water, supplemented with 2 g KH_2PO_4 l^{-1}, named SBM, was used throughout the experiments. Plasmid pDS3154-*inaZ* was previously described by Drainas *et al*. (1995). For plasmid maintenance, chloramphenicol (for suc_{40}/pDS3154-*inaZ*) was added at 100 μg ml^{-1} for solid media and 50 μg ml^{-1} for liquid culture media.

Zymomonas mobilis strain CP4 was treated with the mutagen N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) according to previously described methods (Typas and Galani 1992). The cells were centrifuged and the resulting pellet washed in Ringer's solution three times and resuspended in CM. Viable counts were made on appropriate agar plates following serial dilutions. Colonies capable of growing on 40% (w/v) sucrose were isolated and used in subsequent experiments under selective (40% (w/v) sucrose) and non-selective (2% (w/v) glucose) conditions, on both liquid and solid media, for up to 200 generations.

Conjugative transfer of recombinant plasmids in *Z. mobilis* strains was carried out by filter matings as described previously (Afendra and Drainas 1987). The two *E. coli* donor strains, one containing the helper plasmid pRK2013 (Figurski and Helinski 1979) and the other containing the mobilizable recombinant plasmid carrying *inaZ*, were mixed (10^8 cells ml^{-1} of each in 1 ml LB) and incubated for 1 h at 37 °C, before a 2-ml suspension of *Z. mobilis* recipient cells (3×10^8) was added to the mix and incubated further for 5 h at 30 °C. Selection of transconjugant colonies was performed on complete medium plates containing chloramphenicol.

Each *Z. mobilis* strain was grown in 300-ml conical flasks overnight (20 h) at 24 °C (pH 6·0) in the appropriate medium and then used as an inoculum for experiments in the fermenter. Cells were collected by centrifugation (21 250 *g*, 20 min, 24 °C) and washed three times with the corresponding culture medium before inoculation. All *Z. mobilis* strains were grown in steady-state continuous culture at 24 °C, stirred at 150 rev min^{-1} with N_2 sparging and the pH controlled at 6·0 using 1·0 mol l^{-1} NaOH and 1·0 mol l^{-1} HCl. The fermenter was a Bioflo III (New Brunswick Scientific, Edison, NJ, USA) with a culture working volume of 5 l. At least 10 generations were allowed for the establishment of a steady-state condition and this was confirmed daily by measurements of culture biomass, which should remain constant during a steady-state condition (Karagouni 1979).

The composition of sugar beet molasses, which was kindly provided by the Hellenic Sugar Industry (Salonika, Greece), was (g l^{-1}): dry matter, 791·0; sucrose, 456·5; invert sugars, 5·9; miscellaneous organic materials, 11·1; N, 18·1; CaO, 6·6; K_2O, 52·0; KCl, 11·0; Na_2O, 28·2; NO_3^-, 0·6; SO_4^{2-}, 1·5 and ash, 135·2.

Analytical methods

Biomass was estimated by dry weight determination. Cells were centrifuged (21 250 *g*, 10 min, 4 °C), washed twice with distilled water and dried for 48 h at 110 °C (Park and Baratti 1991a). Ethanol was estimated in culture supernatant fluids by gas chromatography, as described by Finn *et al*. (1984).

The ice nucleation assay was based on the droplet freezing technique developed by Vali (1971), with modifications described by Southworth *et al*. (1988). The ice nuclei activ-

ity of samples, directly collected from the culture, was determined by serial dilution into distilled water and recording the freezing events of 10-μl drops on a $-9\,^{\circ}C$ plate. The ice nuclei activity was expressed as the logarithm of ice nuclei per colony-forming unit and was calculated using the 'Ice' nucleation software program (version 1993), which was kindly provided by Professor Lindow (University of California, Berkley, CA, USA).

Samples for lipid analysis were harvested from steady-state cultures of *Z. mobilis* CP4, suc_{40} and suc_{40}/pDS3154-*inaZ*, grown at a dilution rate of $0{\cdot}10\,h^{-1}$, by centrifugation (21 250 *g*, 10 min, 4 °C), washed with distilled water and lyophilized. Lipids were extracted from the samples by the method of Bligh-Dyer, as modified by Kates (1972). The phospholipids were separated and purified by two-dimensional thin-layer chromatography on a plate of silica gel G (Merck, Darmstadt, Germany) with (i) $CHCl_3 : CH_3OH : H_20$, 65 : 25 : 4 (v/v) in the first dimension and (ii) $CHCl_3 : CH_3OH : CH_3COOH : H_20$, 75 : 5 : 25 : 1·5 (v/v). Phosphorus estimation was performed according to Bartlett (1959). Fatty acid methylesters were prepared according to Morrison and Smith (1964) and analysed as described previously (Koukkou *et al*. 1990).

Sucrose, fructose and glucose were determined in culture supernatant fluids by high-performance liquid chromatography (Hewlett Packard lit with a refractive index detector (1047 A; Hewlett Packard, Wilmington, DE, USA) with a Kromasil-100 C_{18} column (MZ ANALYSENTECHNIK, Mainz, Germany) 250 × 4 mm, 5 μm). Analysis was carried out isocratically with $CH_3CN : H_2O$ (80% : 20%) at 40 °C and a flow rate of 1 ml min^{-1}.

All points on graphs are the mean of four independent experiments.

Statistical analysis

Statistical analyses were accomplished using the MINITAB statistical package (Minitab Statistical Software, State College, PA, USA). Minimum significant differences were calculated from analysis of variance using the Tukey–Kramer method (Petersen 1985; Fry 1989).

RESULTS

Construction of sucrose-hypertolerant suc_{40}/pDS3154-*inaZ*

Over 10 000 independent colonies of MNNG-treated *Z. mobilis* CP4 cultures were isolated and tested for their ability to grow on elevated concentrations of sucrose (30, 35 and 40% (w/v)). However, of the 32 colonies capable of growing on 30% sucrose CM, only 15 could grow on 35% and five on 40% sucrose. When these colonies were tested for their ability to grow at the same rates and with the same phenotypic stability on selective (40% (w/v) sucrose) and non-selective (2% (w/v) glucose) solid media, only two were completely stable, one 30% and one 40% tolerant. Since one of the aims of this project was the utilization of sugar beet molasses for the development of added-value products, the latter mutant was chosen for all subsequent studies.

Plasmid pDS3154-*inaZ* was transferred in suc_{40} from *E. coli* DH5α donors by triparental conjugation. Transconjugants were verified by plasmid isolation back transformation, restriction and Southern blotting hybridization. Plasmid pDS3154-*inaZ* was stable in transconjugants for at least 80 cell cycles, under selective conditions.

Phospholipid and fatty acid content of *Zymomonas mobilis* strains in steady-state continuous culture using complete sucrose medium

Zymomonas mobilis strains grown at $D = 0{\cdot}10\,h^{-1}$ under the same environmental conditions resulted in marked differences in phospholipid and fatty acid composition. The phospholipid content of the wild-type CP4 strain was similar to that reported by Carey and Ingram (1983). In comparison, the mutant strains had a higher content of phosphatidylcholine (PC) and cardiolipin (C), with lower

Table 1 Phospholipid composition of *Zymomonas mobilis* strains*

	CP4	suc_{40}	suc_{40}/pDS3154-*inaZ*
Phosphatidylethanolamine	60·9 ± 5·5	56·6 ± 4·2	51·5 ± 5·8
Phosphatidylglycerol	23·2 ± 0·1	20·7 ± 2·0	25·5 ± 2·4
Cardiolipin	1·7 ± 0·7	6·1 ± 3·5	5·4 ± 2·0
Phosphatidylcholine	9·9 ± 2·4	15·6 ± 2·3	12·8 ± 2·7
Not identified	4·4 ± 1·7	–	3·5 ± 2·9

*All strains were grown in complete sucrose medium, dilution rate $D = 0{\cdot}10\,h^{-1}$, $t = 24\,^{\circ}C$ and pH = 6·0. The values are given as percentage of total phosphorus calculated as the average of measurements from four independent cultures Standard errors were calculated by common numerical analysis.

–, Not detected.

Table 2 Fatty acid composition of *Zymomonas mobilis* strains*

Fatty acids	CP4	suc_{40}	suc_{40}/pDS3154-*inaZ*
14 : 0	3·8 ± 0·9	3·7 ± 0·6	4·2 ± 0·4
14 : 1	7·8 ± 1·4	0·6 ± 0·1	0·2
15 : 0	0·5 ± 0·2	0·5 ± 0·3	0·5 ± 0·3
16 : 0	9·0 ± 0·9	12·9 ± 1·5	12·7 ± 1·4
16 : 1	1·4 ± 0·0	–	–
18 : 0	–	0·4 ± 0·2	0·9 ± 0·5
18 : 1	77·1 ± 1·7	82·8 ± 1·9	81·4 ± 1·7
SFA : UFA	0·15	0·20	0·22

*All strains were grown in complete sucrose medium, dilution rate $D = 0{\cdot}10\,h^{-1}$, $t = 24\,^{\circ}C$ and pH = 6·0. Figures are presented as average of measurements from four independent cultures. Standard errors were calculated by common numerical analysis.
SFA : UFA, Ratio of saturated fatty acids over unsaturated fatty acids; –, not detected.

phosphatidylethanolamine (Table 1). As expected, vaccenic acid (18 : 1) was the main fatty acid constituent in all *Z. mobilis* strains. The content of myristoleic acid (14 : 1) and palmitoleic acid (16 : 1), of the mutant strains, was strongly reduced compared with the wild type, whereas the palmitic acid (16 : 0) content was increased (Table 2). These changes were expressed as the saturated : unsaturated ratio, which increased slightly from 0·15 to 0·22.

Effect of dilution rate on biomass and sugar concentration of *Zymomonas mobilis* strains in steady-state continuous culture

Steady-state cultures of the three *Z. mobilis* strains were obtained between dilution rates of 0·04 and $0{\cdot}14\,h^{-1}$. Below a dilution rate of $0{\cdot}04\,h^{-1}$ washout of the culture occurred and it was not possible to obtain steady-state conditions, mainly due to sugar limitation and possibly to a second nutrient limitation, at these very low growth rates. Culture biomass, as measured by dry weight, remained relatively constant at dilution rates up to $0{\cdot}10\,h^{-1}$, thereafter declining as the dilution rate reached the critical dilution rate (D_{crit}). Culture washout occurred at dilution rates above $0{\cdot}14\,h^{-1}$.

Differences in D_{crit} values were observed between the wild-type, the suc_{40} and the plasmid-containing strain (wild-type $D_{crit} \geq \mu_{max} = 0{\cdot}14\,h^{-1}$ as compared with 0·13 h^{-1} for strain suc_{40} and suc_{40}/pDS3154-*inaZ*). The steady-state biomass concentration was approximately 19% lower for strain suc_{40}/pDS3154-*inaZ* between dilution rates of 0·04 and $0{\cdot}12\,h^{-1}$ under the same growth conditions, compared with the wild-type strain (Fig. 1). Residual sugar concentrations showed the normal nutrient-limited chemostat pattern for both media; below $D = 0{\cdot}10\,h^{-1}$ residual sugar concentrations were very low for all strains but, above $D = 0{\cdot}12\,h^{-1}$, a sharp increase was observed (Fig. 1).

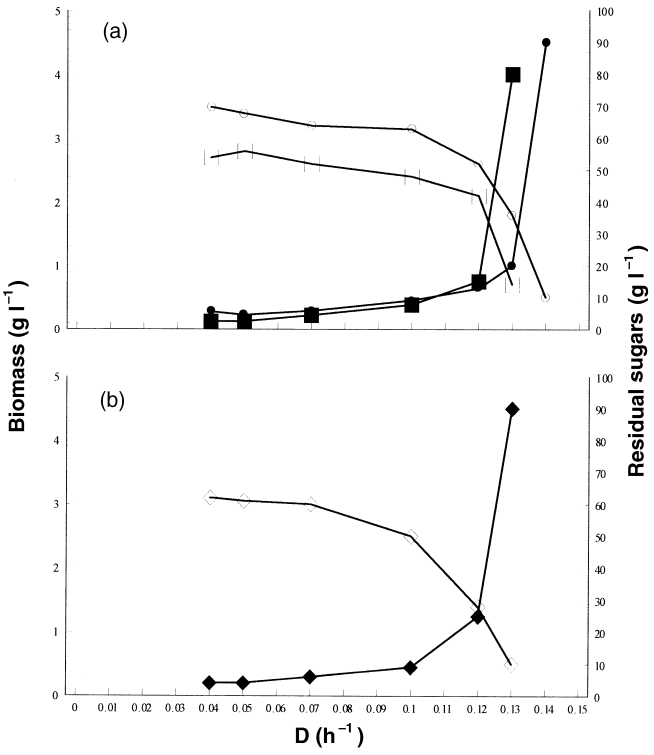

Fig. 1 (a) Effect of dilution rate on *Zymomonas mobilis* strains steady-state biomass (○, CP4; □, suc_{40}/pDS3154-*inaZ*) and residual sugar concentration (●, CP4; ■, suc_{40}/pDS3154-*inaZ*) when both strains were grown in continuous culture containing complete sucrose medium. (b) Effect of dilution rate on *Z. mobilis* suc_{40}/pDS3154-*inaZ* steady-state biomass (◇) and residual sugar concentration (◆) when grown in continuous culture containing SBM. All points on graphs are the mean of four independent experiments with error probability below 0·05

In a 20% (w/v) solution of sugar beet molasses, the wild-type CP4 did not grow over the range $D = 0{\cdot}04$–$0{\cdot}14$ h^{-1} and it was impossible to produce any steady-state condition. Conversely, strains suc_{40} and suc_{40}/pDS3154-*inaZ* achieved steady-state growth between $D = 0{\cdot}04$ and $0{\cdot}13$ h^{-1}. The biomass concentration for strain suc_{40}/pDS3154-*inaZ* remained relatively constant below $D = 0{\cdot}07\,h^{-1}$ (Fig. 1). Thereafter, it decreased gradually as the dilution rate was increased. The biomass values were almost 9% higher at low dilution rates compared with the corresponding values obtained on CSM (Fig. 1). Residual sugar concentrations in both spent culture media were similar. However, below $D = 0{\cdot}10\,h^{-1}$ residual sugar concentrations for all strains were very low. At dilution rates above $0{\cdot}10\,h^{-1}$, a sharp increase in steady-state residual sugars was observed (Fig. 1).

Influence of dilution rate on the ethanol production of *Zymomonas mobilis* strains in steady-state continuous culture

In CSM, ethanol production followed the steady-state biomass curve for all strains. The wild-type strain and strains suc_{40} and suc_{40}/pDS3154-*inaZ* displayed almost constant ethanol production (43 g l^{-1}) between $D = 0{\cdot}04$ and $0{\cdot}10$ h^{-1} (Fig. 2). Thereafter, an 80% decrease in ethanol production occurred. When sugar beet molasses was used as the growth medium (Fig. 2), both strains (suc_{40} and suc_{40}/pDS3154-*inaZ*) produced exactly the same amount of ethanol at each single steady state between $D = 0{\cdot}04$ and $0{\cdot}13$ h^{-1}.

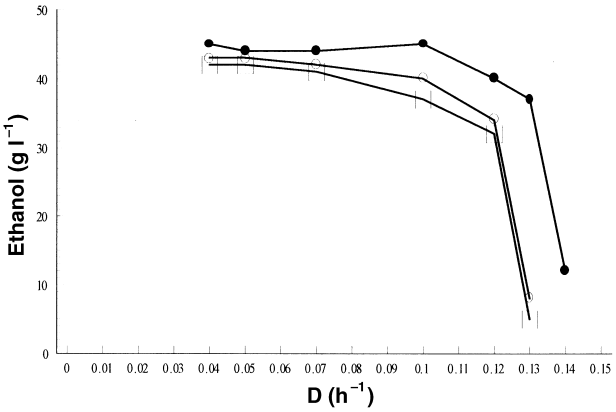

Fig. 2 Effect of dilution rate on steady-state ethanol production by *Zymomonas mobilis* CP4 (●) and suc_{40}/pDS3154-*inaZ* (○) grown in complete sucrose medium and suc_{40}/pDS3154-*inaZ* ethanol production (□) grown in SBM. All points on graphs are the mean of four independent experiments with error probability below 0·05

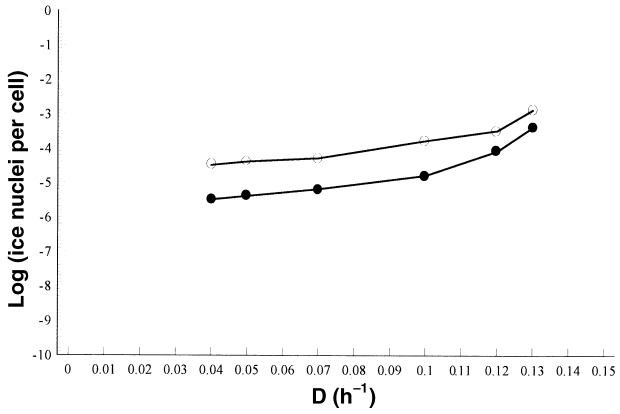

Fig. 3 Effect of dilution rate on steady-state ice nucleation activity of suc_{40}/pDS3154-*inaZ* grown in complete sucrose medium (○) and SBM (●). All points on graphs are the mean of four independent experiments with error probability below 0·05

Influence of dilution rate on ice nuclei activity for suc_{40}/pDS3154-*inaZ*

In addition to ethanol production, ice nucleation activity was studied at different growth rates (Fig. 3). This increased with dilution rate. When the organism was grown in sugar beet molasses, the ice nucleation activity was lower for strain suc_{40}/pDS3154-*inaZ* than the values obtained from growth on CSM, at all dilution rates below $0{\cdot}10\,h^{-1}$. The highest value of ice nucleation activity, in SBM, was observed at $D = 0{\cdot}13\,h^{-1}$, i.e. $-3{\cdot}4$ log (ice nuclei per cell).

DISCUSSION

Several *Z. mobilis* CP4 mutants have so far been tested for ethanol production in sugar beet molasses (Park and Baratti 1991a,b) and the highest ethanol production reported was 56 g l^{-1}, by Park and Baratti (1991a). Other workers observed even higher ethanol production (83 g l^{-1}) when sugar cane molasses was used (Doelle *et al.* 1991).

A hypertolerant mutant, suc_{40}, was selected in our laboratories as the fastest growing and most stable strain in liquid medium containing up to 40% (w/v) sucrose (i.e. 20% sugar beet molasses in water). This strain, as well as the plasmid pDS3154-*inaZ*-containing derivative, displayed a different fatty acid and phospholipid profile compared with the wild-type strain. Fatty acid analysis of all strains used in this work showed the presence of high levels of long-chain unsaturated fatty acids (vaccenic acid (18 : 1)), which was even greater in the mutant strain (about 80%).

Carey and Ingram (1983) suggested that the presence of vaccenic acid, in particular, may explain the ability of this organism to grow in high ethanol concentrations, due to the ethanol destabilizing effect on membrane structure being compensated by the presence of long-chain unsaturated fatty acids. Substitution of unsaturated (myristoleic acid (14 : 1) and palmitoleic acid (16 : 1)) by saturated fatty acids (palmitic acid (16 : 0)) in the mutant strains would allow phospholipid fatty acid chains in the cell membrane to come closer together and to increase the rigidity of the membrane. In this way, the fatty acid composition may contribute to osmotolerance in culture media containing high sugar concentrations. Additionally, increased PC and C in the two mutant strains stabilizes the lipid bilayer (Russell 1989). Therefore, the ability of the mutant and recombinant plasmid-containing strains to grow on high sugar concentrations and in high osmotic pressure environments (molasses) can be attributed to their phospholipid and fatty acid contents.

Plasmid pBZIP1 performed best with respect to ice nuclei production in strain *Z. mobilis* CP4 grown in batch cultures (Varsaki *et al*. 1998). Chloramphenicol added for the plasmid maintenance of pDS3154-*inaZ* (Drainas *et al*. 1995) interfered less with ethanol production in continuous culture when compared with the effects of tetracycline in pBZIP1 (data not shown). The ice nucleation activity of strain suc_{40}/pDS3154-*inaZ* grown in continuous culture in CSM was slightly higher than the activity of cells grown in SBM ($-2{\cdot}9$ *vs* $-3{\cdot}4$ log (ice nuclei per cell)). The corresponding activity of strain suc_{40}/pBZIP1 was 2·5-fold higher than the activity of strain suc_{40}/pDS3154-*inaZ* grown in SBM (data not shown). However, the elevated ice nucleation activity of both strains at the highest dilution rate, irrespective of the substrate (Fig. 3), suggested that it was due to growth rate rather than substrate quality.

In conclusion, the results presented in this paper are considered satisfactory for simultaneous ethanol and bacterial ice nuclei production in *Z. mobilis* and further work is now in progress to improve productivity.

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