An investigation into the preservation of microbial cell banks for alpha-amylase production during 5L fed-batch Bacillus licheniformis fermentations

An investigation into the preservation of microbial cell banks for α-amylase production during 5L fed-batch *Bacillus licheniformis* fermentations.

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Abstract

Fluorescent staining techniques were used for a systematic examination of methods used to cryopreserve microbial cell banks. The aim of cryopreservation here is to ensure subsequent reproducible fermentation performance rather than just post thaw viability. It is clear that *Bacillus licheniformis* cell physiology post thaw is dependent on the cryopreservant (either, Tween 80, glycerol or dimethyl sulphoxide) and that whilst this had a profound effect on the length of the lag phase, during subsequent 5L fed-batch fermentations, it had little effect on maximum specific growth rate, final biomass concentration or α-amylase concentration. Tween 80 was found not only to protect the cells during freezing but also to help them recover post thaw resulting in shorter process times.

Introduction

Within the fermentation industry there is a need to understand and optimise current methods for the preservation of microbial cell banks. From the production of yoghurt to the most advanced recombinant therapeutics, the generation and subsequent maintenance of an optimal cell bank is a critical task in the successful commercialisation of any product made by fermentation (Gnoth *et al*., 2007; Hornbæk *et al*., 2002, 2004; Webb *et al*., 1993). It is therefore surprising that relatively little systematic work has been published concerning the choice of preservation methods and their optimisation. Some generally early work (Lovelock *et al*., 1959; Polge *et al*., 1949; Morgan *et al.,* 2006) has resulted in the formulation of two routinely used preservation methods, both of which are largely unsubstantiated with respect to final productivity. Both freeze-drying and cryogenic preservation rely on the prevention of the formation of ice crystals (Fuller, 2004; Hubálek, 2003; Kirsop and Snell 1984), a

process known to disrupt the delicate membrane structures within cells and hence compromise their viability. Starting from a liquid suspension of cells or spores, freeze drying involves the sublimation of water at low temperatures and pressures and is generally considered inferior to cryogenic preservation, where cells are suspended in an appropriate medium supplemented with 20% v/v glycerol and rapidly frozen at -80°C (Polge *et al*., 1949). A brief literature search shows that there are few variations on these themes. Other cryoprotectants, such as Tween 80 (Beal *et al*., 2001; Endo *et al*., 2006; Smittle *et al*., 1974), yeast and malt extract (Hubálek, 2003; Johannsen, 1972) and more defined agents such as DMSO (Lovelock *et al*., 1959) have also been studied. However, there is little informed or systematic work to back up their use, while the current industrial practice of testing working cell banks through productivity measurements in small scale process representations of production scale often leads to the discovery of a poor cell bank which must then be discarded. Therefore it would seem that there is a real need for a systematic examination of the methods used to preserve cell banks to ensure subsequent reproducible fermentation performance. Measurable effects on 'sensitivity', defined here as a loss of productivity rather than just cell viability, may be subtle so appropriate techniques to measure the effects are required.

Multi-parameter flow cytometry has many advantages over conventional microbiological analyses such as dilution plating (c.f.u. per ml) and these have been extensively reviewed elsewhere (Nebe-von-Caron *et al.*, 2000; Hewitt and Nebe-von-Caron 2001, 2004). Briefly, using various mixtures of fluorescent dyes, it is possible to resolve an individual microbial cell's physiological state beyond culturabilty, in 'real-time', based on the presence or absence of an intact polarised cytoplasmic

membrane and the transport mechanisms across it. The presence of both an intact polarised cytoplasmic membrane and active transport are essential for a fully functional (healthy) cell. The speed and sensitivity of this technique was used here to discover those preservation/recovery combinations resulting in the correct physiological state post thaw, which could correlate to final process productivity. In this way candidate preservation/recovery methods were investigated and tested on a standard process model, namely the use of *Bacillus licheniformis* to produce the commercially important enzyme α-amylase (Hewitt and Solomons 1996) in 5L fed-batch fermentation processes.

MATERIALS AND METHODS

Organism and growth conditions.

Cell banks of an industrial production strain (Novozymes A/S, Bagsværd, Denmark) of *B. licheniformis* SJ 4628, an asporulating alpha-amylase overproducing strain, were maintained in 1 mL vials at an OD_{600nm} of 1 at -80^{o}C in Yeast Malt Extract Broth (Oxoid, UK) supplemented with 20% v/v glycerol, 25% v/v glycerol, 15% v/v DMSO or 20% v/v Tween 80.

Inocula were prepared by diluting a 1 mL aliquot from the cell bank into 9 mL M9 buffer (composition gL^{-1}: KH_2PO_4, 3; Na_2HPO_4, 6; NaCl, 5) made up in distilled water. 1 mL of 1M $MgSO_4$ was added after sterilisation. The cells were then grown in a static 500 mL Erlenmeyer flask on 50 mL yeast malt agar (composition gL^{-1}: malt extract powder, 40; yeast extract powder, 20; M Lab agar powder, 20) made up in distilled water. After 16 h of incubation at 37 °C, 20 mL of M9 buffer was added aseptically to the surface of the agar and the culture mixed to re-suspend the cells.

For shake flask toxicity experiments cells were grown in 50 mL of yeast malt broth in a 500ml baffled Erlenmeyer shake flasks at 37°C and 200rpm. For all shake flask experiments a 2% inoculum (prepared from cryopreserved stocks as above) with an optical density at 600 nm of 1.0 was used.

For laboratory scale fed-batch experiments cells were grown in yeast malt extract medium (composition gL^{-1}: $(NH_4)_2HPO_4$, 16; malt extract powder, 40; yeast extract powder, 20; glucose, 5; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.45; $MnSO_2.4H_2O$, 0.04; $ZnSO_4.7H_2O$, 0.02; $FeSO_4.7H_2O$, 0.02; $Na_2MoO_4.2H_2O$, 0.002; $CuSO_4.5H_2O$, 0.01) made up in distilled water with 3 mL of polypropylene glycol 2025. A feed solution (composition gL^{-1}: $(NH_4)_2HPO_4$, 16; malt extract powder, 40; yeast extract powder, 20; glucose, 500; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.45; $MnSO_2.4H_2O$, 0.04; $ZnSO_4.7H_2O$, 0.02; $FeSO_4.7H_2O$, 0.02; $Na_2MoO_4.2H_2O$, 0.002; $CuSO_4.5H_2O$, 0.01) was made up in distilled water. The fed batch fermentations were carried out in a 5 L cylindrical glass bioreactor (160 mm diameter and 300 mm total height), with a working volume of 4 L. The vessel was fitted with two 82 mm, six flat bladed paddle type impellers which were 80 mm apart, with the lowest impeller situated 80 mm from the bottom of the vessel. The vessel was also fitted with four 90° baffles of 15 mm width spaced equidistantly around the vessel. The fermentation vessel was equipped for the measurement of DOT, pH and temperature. DOT was maintained at 90% saturation by varying the impeller speed from 0 to 1000 rpm, pH was maintained at 7.0 by addition of 2 M NaOH and 2 M H_3PO_4 on demand and temperature was maintained at 37 °C. Foaming was controlled by addition of 2% polypropylene glycol 2025 on demand. For all fed batch fermentations a 5% inoculum was used with an optical density of 1.0. Fed batch

fermentations were started as batch cultures and an exponential feeding profile was calculated from the following equation (Strandberg *et al*., 1994),

$$F = \left(\frac{1}{s}\right) \times \left(\frac{\mu}{Y_{xs}} + M\right) \times X_o \times e^{\mu t}$$

Where S= 500g/L, $Y_{x/s}$ = 0.5, m= 0.02 and Xo =5g and μ = 0.3h^{-1}.

Feeding was started when the initial glucose was exhausted as measured by a sudden rise in DOT (spike). When the DOT reached the 20% saturation level the feed rate was held constant for the remainder of the experiment.

Analytical Techniques

Biomass was measured turbidimetrically by optical density at 600 nm in a double beam spectrophotometer and measurements of dry cell weight, gL^{-1} and plate counts, c.f.u. mL^{-1} were taken. Glucose concentration was measured using a Cecil HPLC system with an AminexHPX-87P carbohydrate analysis column (Bio-rad, UK) with a Cecil 4700 refractive index (RI) analyser (Cecil Instruments, Cambridge, UK). α-amylase concentration was determined spectrophotometrically using the method determined by the International Federation of Clinical Chemistry and Laboratory Medicine (Lorentz, 1998).

Fluorescent measurements were made using a Coulter (High Wycombe, UK) EPICS ELITE flow cytometer with 488 nm excitation from an argon-ion laser at 15 mW. Samples taken from the culture were immediately diluted (at least 1:2000 v/v) with

phosphate buffer solution (PBS, pH 7.0) and stained with a mixture of propidium iodide and bis-(1, 3-dibutylbarbituric acid ($DiBAC_4(3)$) Samples were kept in a sonication bath for 10 s prior to analysis, in order to avoid problems associated with cell aggregation. Stock solutions of each dye were prepared as follows: $DiBAC_4(3)$, was made up at 10 mg ml^{-1} in DMSO and PI was made up at 2 mg ml^{-1} in distilled water. The working concentrations of $DiBAC_4(3)$ and PI were 0.6 μg ml^{-1} and 3 μg ml^{-1}, respectively in Dulbecco's buffered saline (pH 7.2, DBS). All solutions were passed through a 0.2 μm filter, immediately prior to use, to remove particulate contamination. Additionally, software discriminators were set on both the forward and right angle light scatter signals to further reduce electronic and small particle noise. The optical filters were set up so that PI fluorescence was measured at 630 nm and BOX fluorescence at 525 nm. Where there was spectral overlap the systems software compensation was set up to eliminate any interference

RESULTS AND DISCUSSION

Shake flask experiments were carried out using *B. licheniformis* SJ 4628 inocula derived from different cryopreserved cell banks namely 20% v/v glycerol, 25% v/v glycerol, 15% v/v DMSO or 20% v/v Tween 80. Cell banks frozen without a cryopreservant were also used as a control. In all cases cell banks had >98% viability (as measured by PI exclusion) prior to freezing. Triplicate reproducible measurements of OD_{600nm} were made (Figure 1) and cell physiology was monitored throughout the fermentations using multi-parameter flow cytometry (Figure 2). In order to look at the effect of cryopreservant on α-amylase productivity further fed-batch fermentations were carried out in 5 L bioreactors by growing the same cell banks, with and without

the 16 h incubation pre-inoculation step on yeast malt extract agar, to an $OD_{600\ nm}$ of 1.0 prior to inoculation. Duplicate reproducible measurements of OD_{600nm}, DCW (gL^{-1}), $CFUmL^{-1}$, glucose concentration (gL^{-1}) and α-amylase concentration ($\mu KatL^{-1}$) were made (Figure 3, not all data shown). Cell physiology was monitored throughout the fermentation using multi-parameter flow cytometry (Figure 4).

During the shake flask fermentations (Figure 1) both the lag phase and subsequent cell physiology (Figure 2, not all data shown) were affected by the choice of cryopreservant used. The cell banks prepared with 20% v/v Tween 80 had the shortest lag phase (~ 3.5 h) presumably because Tween 80 provides better protection from the damaging effects of freezing than the other cryopreservants. Oleic acid is a component of the Tween 80 molecule and has been shown to enhance the ratio of unsaturated fatty acids in the cytoplasmic membrane of lactic acid bacteria allowing them to resist damage during freezing (Beal *et al*., 2001; Endo *et al*., 2006; Smittle *et al*., 1974). Oleic acid is considered necessary for the formation of the cytoplasmic membrane and studies performed with various *Lactobacillus spp.* strains showed that growth was stimulated in the presence of Tween 80 (Endo *et al*., 2006). Cell banks prepared with 15% v/v DMSO showed the longest lag phase (~ 8.5 h) indicating that DMSO is not as efficient at protecting cells during freezing when compared with the other cryoprotective compounds, or had some alternative deleterious effect. The 'beneficial' cryoprotective effects of DMSO were discovered early in the science of cryopreservation and it has since become a popular cryoprotective agent (Lovelock *et al*., 1959). It is claimed that DMSO protects cells by entering the cytoplasmic membrane and directly interacting with the lipid bilayer. Indeed, DMSO has been shown to increase membrane fluidity which is consistent with positive cryoprotective

effects (Gurtovenko *et al*., 2007). These benefits would not seem to have been reproduced here. Interestingly, the cell banks prepared with 20 % v/v glycerol and 25% v/v glycerol show similar lag phase lengths when compared with cell banks prepared with no additional cryoprotectant (~ 4 h). Glycerol was also an early development in the science of cryopreservation and since then has been used widely to protect cells during freezing (Polge *et al*., 1949). As a small poly hydroxylated molecule glycerol can enter cells and hydrogen bond to water molecules (Fuller, 2004) preventing ice crystal formation. It is also less toxic to cells than DMSO (Simione, 1998). These results suggest that the addition of glycerol to the yeast malt extract medium surrounding the cells has little effect. This may be because yeast and malt extract themselves are known to be good cryoprotectants for lactic acid bacteria and are often included in freezing protocols (Hubálek, 2003; Johannsen 1972). The medium used for these experiments also contained polypropylene glycol 2025 (as an antifoam) which may have helped to protect the cells as both propylene glycol and ethylene glycol have been shown to work as effective cryopreservants (Hubálek, 2003). Surprisingly, the degree of cell death during the freezing process as measured by PI positive staining did not correlate well with length of lag phase. For example 15% v/v DMSO had the same degree of cell death (~47%) post thaw, as 2% v/v glycerol although it took longer for the culture to recover (Figure 5). Indeed there was a considerable proportion of cells (~40%) having depolarised cytoplasmic membranes (i.e. $DiBAC_4(3)$ positive) 2 hrs after incubation in the growth medium. This was not observed in any other case but is consistent with a longer lag phase in the case of 15% v/v DMSO. Further, 20% v/v Tween 80 had a higher degree of cell death (~ 20% post thaw) than 25% v/v glycerol (~13% $DiBAC_4(3)$) with the degree of death in cell banks with no cryopreservant being ~38%. In all cases the viable population outgrew

the dead cell population and the cells were all viable when the culture reached an OD_{600nm} of 1.0 although the length of time to get there was different in each case. It is clear therefore that there isn't a simple correlation between cell viability post thaw and length of lag phase; the freezing effects are more complicated than that.

When each of the shake flask cultures derived from the different cryopreserved stocks reached an OD_{600nm} of 1.0 they were used to inoculate 5L fed batch fermentations to determine if the method of cryopreservation affected fermentation process performance as indicated by yield of α-amylase (Figure 3). In all cases no difference was seen in terms of overall growth characteristics (max DCW ~ 27 g L^{-1} at 17 h), cell physiological state or concentration of α-amylase (max ~10^6 μKat L^{-1} at 50 h) when using inocula derived from each of the different cell banks and with or without the overnight incubation step on yeast extract malt extract agar (Figures 4 and 5, not all data shown). It is clear therefore that in this case inoculum pre-history had no bearing on final process outcome, despite the use of the same number of propagation steps as exploited at the larger scale. However, it is recognised that this may not always be so. *B. licheniformis* SJ4628 as an industrial production strain is extremely robust in nature and the *AmyE* gene which encodes for the α-amylase protein is located on the chromosome of the organism rather than on a plasmid. So plasmid instability, which may be affected by cryopreservation, was not an issue here. Other commercially important systems which use plasmid technology may be more sensitive to cryopreservation technique so the results presented here should be used cautiously.

CONCLUSIONS

It is not unreasonable to conclude that any of the cryopreservation techniques described here could be used for productive industrial fermentations of *B. licheniformis* SJ4628 and probably other *Bacillus* spp. too. It is also recognised that there will be little motivation to introduce a new cryopreservation protocol if the existing protocol is adequate, especially since this work has demonstrated that it is unlikely that poor cryopreservation is responsible for poor working cell banks which do not have the same process performance as the key strain. So, provided the inoculum is grown up to the same OD and percentage viability, the subsequent production process ought to be robust. It is therefore more likely that the physiological condition or history of the organisms prior to preservation is responsible for the incidence of poor working cell banks. However, if difficulties are being experienced with a particular cryopreservation protocol then Tween 80 may be worth consideration, as it not only protects the cells during freezing but also helps them to recover post thaw resulting in shorter process times.

NOMENCLATURE

F feed rate (Lh^{-1})

S substrate concentration in the feed solution (gL^{-1})

μ desired specific growth rate (h^{-1})

Y_{xs} maximum biomass yield with a limiting substrate (g/g)

X_0 initial amount of cells at the start of feeding (g)

m maintenance coefficient ($gg^{-1}h^{-1}$)

t time after feeding commences (h)

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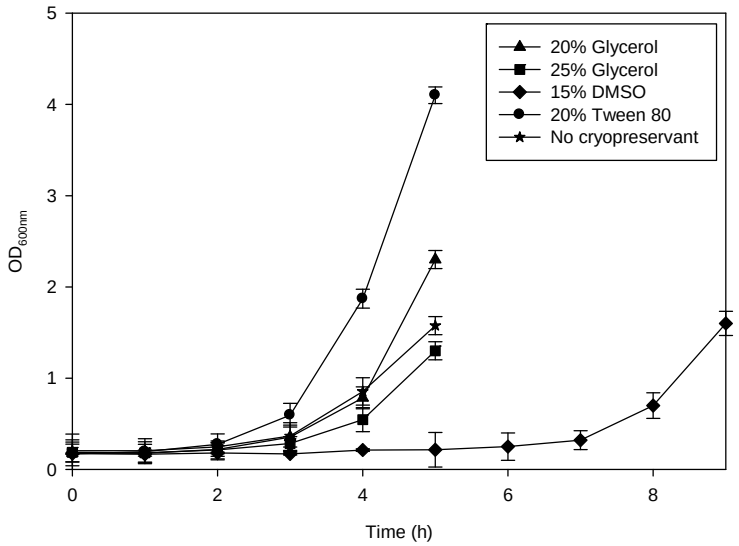

20% Glycerol
25% Glycerol
15% DMSO
20% Tween 80
No cryopreservant
OD600nm
Time (h)
0
1
2
3
4
5
0
2
4
6
8

Figure 1.

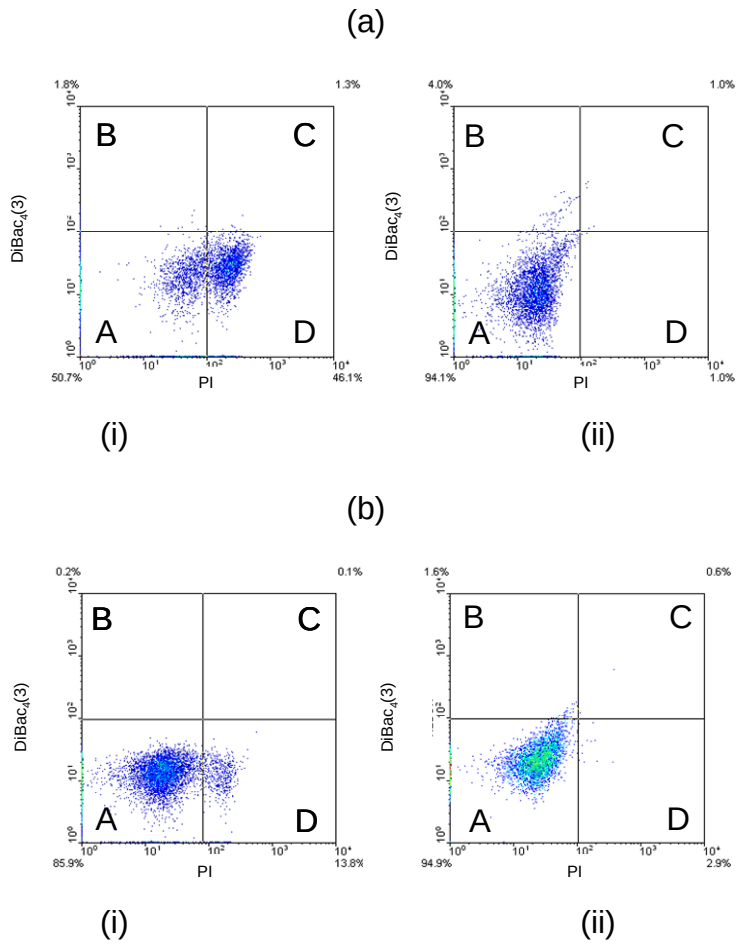
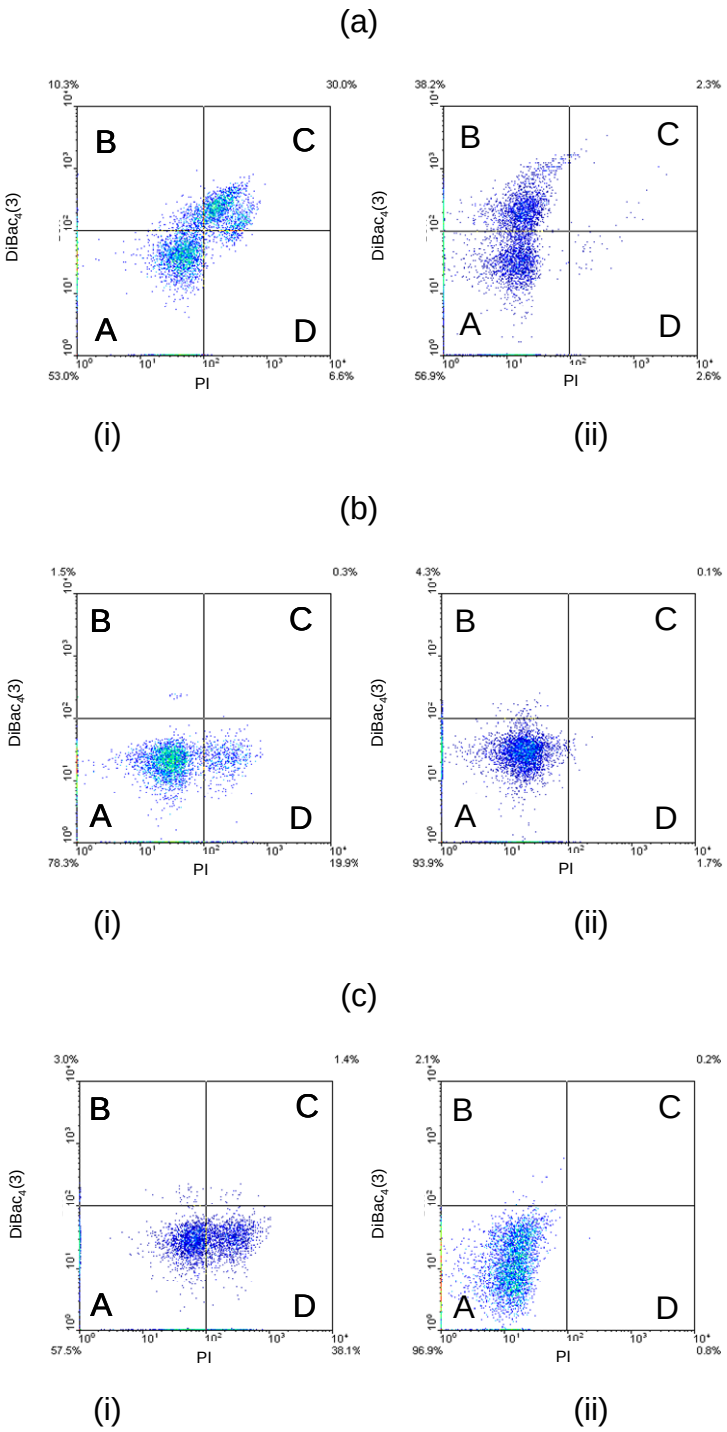

(a)
1.8%
1.3%
B
C
A
D
50.7%
46.1%
DiBac4(3)
PI
(i)
4.0%
1.0%
B
C
A
D
94.1%
1.0%
DiBac4(3)
PI
(ii)
(b)
0.2%
0.1%
B
C
A
D
85.9%
13.8%
DiBac4(3)
PI
(i)
1.6%
0.6%
B
C
A
D
94.9%
2.9%
DiBac4(3)
PI
(ii)

Figure 2.


(a)
10.3%
30.0%
B
C
A
D
53.0%
6.6%
DiBac4(3)
PI
(i)
38.2%
2.3%
B
C
A
D
56.9%
2.6%
DiBac4(3)
PI
(ii)
(b)
1.5%
0.3%
B
C
A
D
78.3%
19.9%
DiBac4(3)
PI
(i)
4.3%
0.1%
B
C
A
D
93.9%
1.7%
DiBac4(3)
PI
(ii)
(c)
3.0%
1.4%
B
C
A
D
57.5%
38.1%
DiBac4(3)
PI
(i)
2.1%
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B
C
A
D
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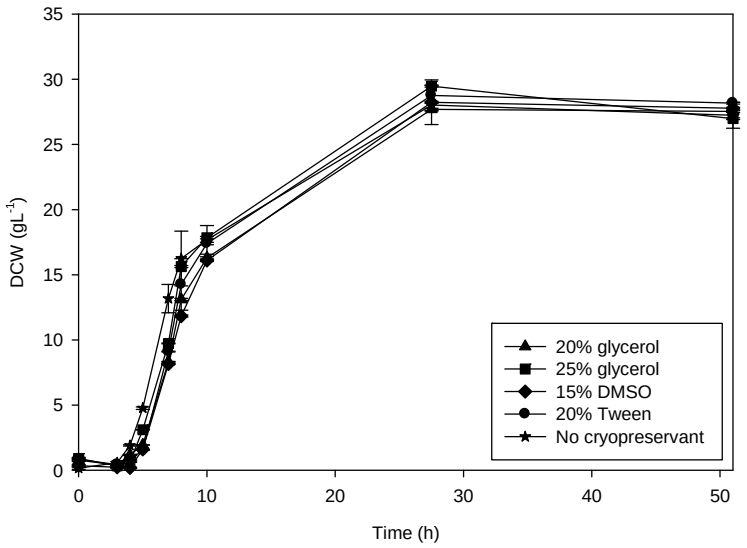
DiBac4(3)
PI
(ii)

Figure 3.

(a)


DCW (gL-1)
Time (h)
20% glycerol
25% glycerol
15% DMSO
20% Tween
No cryopreservant

(b)

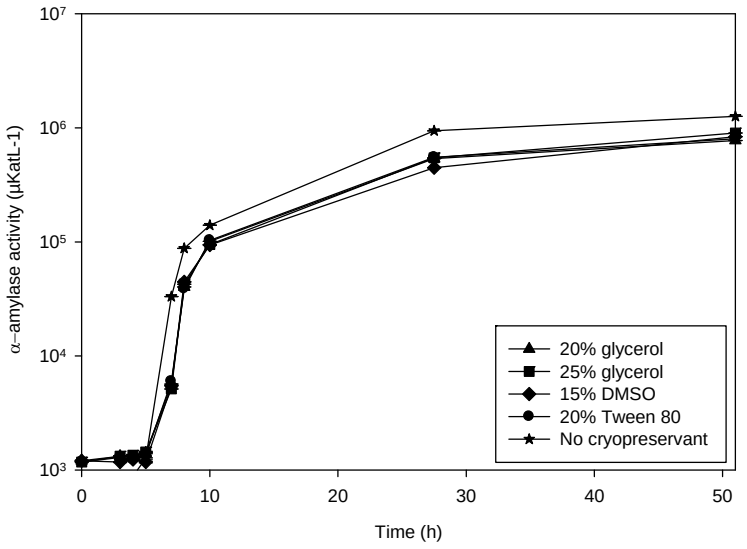

α–amylase activity (µKatL-1)
Time (h)
20% glycerol
25% glycerol
15% DMSO
20% Tween 80
No cryopreservant

Figure 4.

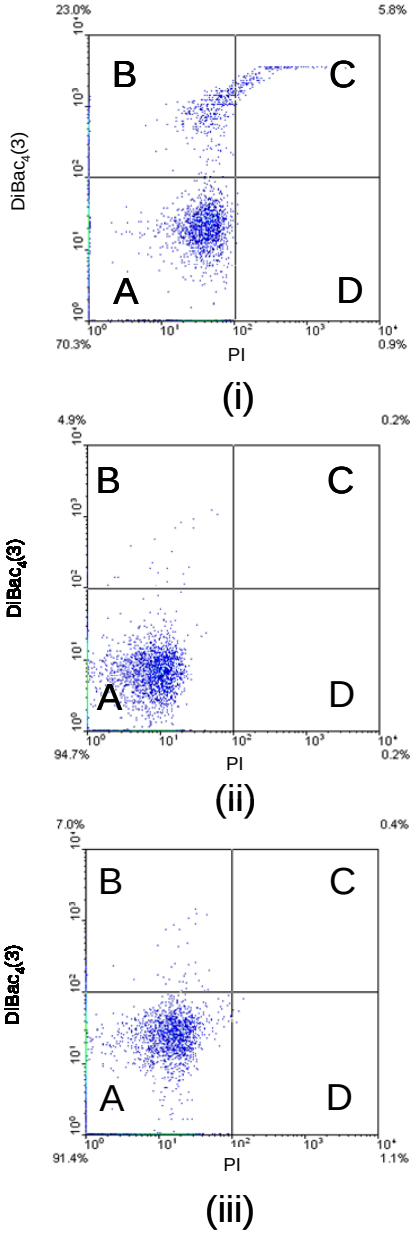

23.0%
5.8%
B
C
A
D
70.3%
0.9%
DiBac4(3)
PI
(i)
4.9%
0.2%
B
C
A
D
94.7%
0.2%
DiBac4(3)
PI
(ii)
7.0%
0.4%
B
C
A
D
91.4%
1.1%
DiBac4(3)
PI
(iii)

Figure 5.

Figure 1. OD_{600nm} profile for *Bacillus licheniformis* SJ4628 during shake flask fermentations post thaw following cryopreservation with either 20% v/v glycerol, 25% v/v glycerol, 15% v/v DMSO, 20% v/v Tween 80 or no cryopreservant as a negative control. Error bars represent the range of data collected from three replicates.

Figure 2. Flow cytometric analysis of *Bacillus licheniformis* SJ4628 stained with $DiBAC_4(3)$/PI taken at 0 h (i) and 2 h (ii) during shake flask fermentations post thaw following cryopreservation with: (a) 20% v/v glycerol and (b) 25% v/v glycerol. Up to four subpopulations can be identified, these correspond to cells with an intact polarised cytoplasmic membrane, not stained (A), cells with an intact depolarised cytoplasmic membrane, stained with $DiBAC_4(3)$ (B), cells with a permeablised depolarised cytoplasmic membrane stained with PI/ $DiBAC_4(3)$/and(C)/or PI only (D).

Figure 3. Flow cytometric analysis of *Bacillus licheniformis* SJ4628 stained with $DiBAC_4(3)$/PI taken at 0 h (i) and 2 h (ii) during shake flask fermentations in yeast malt broth following cryopreservation with different treatments: (a) 15% v/v DMSO, (b) 20% v/v Tween 80 and (c) no cryopreservant as a negative control. Sub-populations can be identified as in Figure 2.

Figure 4. (a) DCW (gL^{-1}) and (b) alpha-amylase activity ($\mu KatL^{-}1$) profiles for *Bacillus licheniformis* SJ4628 5L fed-batch fermentations post thaw, with the 16 hrs overnight incubation step, following cryopreservation with 20% v/v glycerol, 25% v/v glycerol, 15% v/v DMSO, 20% v/v Tween 80 and no cryopreservant as a negative control. Error bars represent the range of data collected from two replicates.

Figure 5. Flow cytometric analysis of *Bacillus licheniformis* SJ4628 stained with $DiBAC_4(3)PI$ taken at 0 h (i), 3 h (ii) and 4 h (iii) during fed-batch 5L fermentations post thaw after the 16 hrs overnight incubation step following cryopreservation with 20% v/v glycerol. Sub-populations can be identified as in Figure 2.