Sorbitol/methanol mixed induction reduces process impurities and improves centrifugal dewatering in *Pichia pastoris* culture

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Abstract

This study investigates how sorbitol/methanol mixed induction affects fermentation performance, dewatering characteristics of cells during harvesting and the profile of host cell proteins (HCP) in the process fluid when producing the target recombinant protein aprotinin. Compared to standard methanol induction, sorbitol/methanol (1:1, C-mol/C-mol) mixed induction improved cellular viability from 92.8±0.3% to 97.7±0.1% although resulted in a reduced product yield from 1.65±0.03 $g \cdot L^{-1}$ to 1.12±0.07 $g \cdot L^{-1}$. On the other hand, average oxygen consumption rate (OUR) dropped from 241.4±21.3 $mmol \cdot L^{-1} \cdot h^{-1}$ to 145.5 ±6.7 $mmol \cdot L^{-1} \cdot h^{-1}$. Cell diameter decreased over time in the mixed induction, resulting in a D_{50} value of 3.14 μm at harvest compared to 3.85 μm with methanol. The reduction in cell size enhanced the maximum dewatering efficiency from 78.1±3.9% to 84.5±3.3% as evaluated by using an established ultra scale-down methodology that models pilot and industrial scale disc stack centrifugation. Seventy host cell proteins (HCPs) were identified in clarified supernatant when using sorbitol/methanol mixed induction regimen. The total number of HCPs identified with standard methanol induction was nearly one hundred. The downstream process advantage of the mixed induction lies in improved product purity by reducing both cell mortality and level of released whole cell proteins. This needs to be balanced and optimised against the observed reduction in product yield during fermentation.

Highlights

- Sorbitol/methanol induction increases cell viability and decreases oxygen consumption
- The mixed strategy halves the quantity of inflammable methanol needed at scale-up
- The mixed induction reduces number of host cell proteins co-released with the product
- The mixed induction improves the centrifugal dewatering of cell culture

Keywords: *P. pastoris* fermentation, sorbitol/methanol mixed induction, dewatering, host cell proteins.

1. Introduction

P. pastoris is becoming a popular host for the production of heterologous proteins as it has characteristics of both eukaryotic and prokaryotic organisms. As an eukaryote, it contains protein processing machineries to perform protein secretion,

disulphide bond formation and glycosylation [1]. Fully monoclonal antibodies have been reported to be expressed in *P. pastoris* with a titre over 1 g•L^{-1} [2]. Meanwhile, *P. pastoris* has the features of prokaryotes. Like *Escherichia coli*, it exhibits fast growth in minimal medium with a maximum growth rate of 0.26 h^{-1} [3]. Compared to mammalian cells, it has less rigorous nutrient requirements with minimal susceptibility to shear stress and heterogeneity of environment [4].

However, despite these advantages, scale-up of *P. pastoris* cultivation faces challenges in industry. As a methylotrophic yeast, it uses methanol as the inducer of alcohol oxidase 1 promoter (*pAOX1*) [5]. Methanol usage is constrained by high oxygen demand and need for heat removal in large scale bioreactors which impose potential design restrictions [6]. It is reported that 0.8-1.1 mol of O_2 was consumed and 727 kJ heat was generated by *P. pastoris* to metabolize one mole of methanol [7]. Correspondingly, the bioreactor requires a OTR value over 230-290 mmol•L^{-1}•h^{-1} when methanol is fed at the rate recommended by Invitrogen in *Pichia* Fermentation Process Guidelines [8]. However, traditional fermentation bioreactors only have average OTR of 150-200 mmol•L^{-1}•h^{-1} [9]. Besides, using methanol imposes challenge to strict health and safety regulations. Thus, reducing methanol consumption is potentially advantageous to process scale-up.

Partially replacing methanol with sorbitol has been suggested to reduce drawbacks of methanol usage and benefit *P. pastoris* cultivation [10]. Sorbitol has a relatively low enthalpy of combustion and thus sorbitol/methanol mixed

induction could reduce oxygen consumption rate up to two-fold. Besides, sorbitol/methanol mixed induction reduces formation of toxic formaldehyde and enhances cellular viability [11]. Effect of sorbitol/methanol mixed induction on product yield is strain dependent. Celik and co-workers reported that productivity of recombinant human erythropoietin was enhanced 1.8 times by using sorbitol as a one shot addition at the induction time whilst linearly feeding methanol [10]. Niu and co-workers found that product yield of β-galactosidase was comparable when mole fraction of $C_{methanol}$ was maintained in the range of 45% ~100% [12]. However, no report has addressed its impact on product recovery and purification.

In a previous study performed in our laboratory, an ultra scale-down model of pilot and industrial scale centrifuges was established to predict dewatering levels at scale [13]. It was shown that the dewatering levels were affected by the choice of *P. pastoris* strains under pure methanol induction [14].

In the present work, fermentations using pure methanol and sorbitol/methanol mixed inductions were compared. Impact of sorbitol/methanol mixed induction on fermentation and early downstream processing, focusing on product recovery and level of HCPs impurities that influence chromatography steps was investigated.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Poole, UK) unless otherwise specified.

2.2. Yeast strain and culture medium

P. pastoris CLD804 strain expressing recombinant aprotinin was kindly provided by Fujifilm Diosynth Biotechnologies (Billingham, UK). The product expression was under the control of *pAOX1*. Buffered glycerol complex medium was used for cell culture in shaking flask and basal salt medium (BSM) was used in bioreactors. 0.75 g•ml^{-1} sorbitol solution was prepared in Milli-Q water to obtain a solution that has same volumetric carbon numbers as methanol. Sorbitol/methanol (1:1, C-mol/C-mol) mixed solution was prepared by mixing the same volumetric amount of pure methanol and sorbitol solution.

2.3. Cultivation in bioreactor

Multifors-2 benchtop bioreactor (Infors UK Ltd., Reigate, UK) which consists of four one-litre glass vessels was used, and fermentation was performed following the procedure recommended by Invitrogen in *Pichia* Fermentation Process Guidelines Overview [8]. The temperature was set at 30℃ and pH was maintained at 5.0 by adding 15% (v/v) ammonia. The dissolved oxygen (DO) was maintained at 30% throughout the fermentation by controlling the agitation and

air/oxygen mixture. The cultivation was started with cell optical density of 1.0. *P. pastoris* cells were firstly cultured in basal salt medium supplemented with 40 $g \cdot L^{-1}$ glycerol. Complete glycerol depletion was recorded by a DO spike, at which time 50% (v/v) glycerol was fed in at the rate of 18 $ml \cdot L^{-1} \cdot h^{-1}$ until OD_{600} of broth reached 300 (~50 g $DCW \cdot L^{-1}$). The production was then induced by feeding methanol or sorbitol/methanol mixture at constant rate of 10.8 $ml \cdot L^{-1} \cdot h^{-1}$ (270 mmol $carbon \cdot L^{-1} \cdot h^{-1}$). Duplicate fermentations were conducted for both methanol and sorbitol/methanol mixed induction.

2.4. Analytical methods

The dry cell weight (DCW) was used to determine cell density. 1ml of culture sampled from the bioreactor was pipetted into 1.5 ml Eppendorf tube and centrifuged at 4000 g for 10 min using Eppendorf 5415R (Eppendorf UK limited, Stevenage, UK). After the supernatant was removed, the wet pellet was dried at 100℃ for 24 hours and the remaining solid was weighted.

The cellular viability was determined by measuring proportion of cells that were penetrated by propidium iodide. Cell broth was diluted to optical density of 0.05 at 600nm using 0.9% (v/v) NaCl before being stained. Florescence was measured by Accuri™ C6 cytometer (BD Biosciences, Wokingham, UK).

The aprotinin concentration was quantified using the protocol recommended by Sigma-Aldrich in Enzymatic Assay of Aprotinin [20]. The standard curve

between aprotinin concentration and inhibition rate was built using bovine aprotinin.

Electrophoresis assay of the soluble proteins in supernatant was performed in NuPage SDS Novex precast gel with 4–12% gradient (Invitrogen, Paisley, UK). 5 µl of supernatant was loaded in each well and electrophoresis was performed at constant voltage of 200 V for 40 min. After being stained by Quick Coomassie Stain (Generon, Slough, UK), the protein bands were visualized using Amersham Imager 600 (GE Health Care, Amersham Place, UK).

Host cell proteins in the supernatant were identified using a method reported before [21]. The soluble proteins were concentrated by an 20% SDS-PAGE gel and then being chemically digested. The peptide mixture was analysed by electrospray liquid chromatography-mass spectrometry (LC-MS/MS). Spectrum was processed using Proteome Discoverer (Thermo Fisher Scientific Inc.) and searched against Uniprot database using Mascot search algorithm (Matrix Science, London, UK). Protein identification was conducted using Scaffold (Proteome Software Inc., Portland, OR, USA). The identification was considered acceptable if threshold could be established over 95% probability and the protein contained at least one identifiable unique peptide.

2.5. Prediction of centrifugal dewatering

Dewatering level of the cell cultures in disc stack centrifuge was predicted using the method as reported before [13]. Cell cultures were harvested from the

bioreactor and diluted to a volumetric cell fraction of 30% (v/v) using Milli-Q water. Afterwards, 2 ml and 10ml of the cultures were pipetted into 2.2 ml Eppendorf tubes and 15 ml Falcon tubes, respectively. The 2.2 ml and 15 ml tubes were centrifuged by Eppendorf 5810R (Eppendorf UK, Stevenage, UK) and Beckman Coulter Avanti J-E Centrifuge (Beckman Coulter United Kingdom, High Wycombe, UK), respectively, to predict the dewatering in CSA-1 or BTPX-305 disc stack centrifuges, respectively. Dimension, speed and sigma (Σ) of the centrifuges were shown in Table.2. After the supernatant was discarded thoroughly, cell pellets in the tubes were weighted before and after being dried at 100℃ for 24 hours.

3. Theoretical considerations

3.1. Prediction of centrifugal dewatering

Centrifugation speed, residence time and solid heights are critical factors in dewatering of cell culture [15]. To develop a scale-down model, it is necessary to maintain constant relative centrifugal force (RCF). Liquid flow rate determines the residence time of solids in large scale centrifuges. At small batch scale, this can be defined as the ratio of volume to centrifugation time. Solid height determines the pressure applied to the solid which affects dewatering. Thus, a cell concentration that would give same solid heights in both scales should be used in the scale-down experimentation.

Here sigma (Σ) of centrifuges, which considers not only speed and time but dimensions of centrifuges, was used. Sigma theory has been widely used to predict the performance of large scale centrifuges using laboratory benchtop ones [16]. By using Eq. 1, different flow rates at scale can be mimicked by running benchtop centrifuges for different time periods.

$$\frac{Q}{C\Sigma} = \frac{V_{Lab}}{t_{Lab}C_{Lab}\Sigma_{Lab}} \quad (1)$$

where Q is the liquid flow rate in large scale centrifuge, Σ and Σ_{lab} are setting area of large scale and laboratory scale centrifuge, V_{lab} is the volume of sample in laboratory scale tube, t_{Lab} is the setting time of sample in laboratory centrifuge, C and C_{Lab} are correlation factors for deviation of non-ideal liquid in large scale centrifuge and laboratory centrifuge.

For a laboratory scale benchtop centrifuge, Σ_{lab} can be calculated by Eq. 2 [17].

$$\Sigma_{Lab} = \frac{V_{Lab}\omega^2(3 - 2x - 2y)}{6g\ln(\frac{2R_2}{R_2+R_1})} \quad (2)$$

where ω is the angular velocity of centrifuge, R_2 and R_1 are outer and inner radius of centrifuge rotor, x and y are fractional time of acceleration and deceleration of centrifuge, g is the gravitational acceleration.

For a disc stack centrifuge, Σ_{Ds} can be calculated by Eq. 3 (Boychyn et al. 2004).

$$\Sigma_{Ds} = \frac{2\pi n\omega^2(R_2{}^3 - R_1{}^3)}{3g\tan\theta} \quad (3)$$

where n is the disc numbers, θ is the half disc angle.

3.2. Calculation of dewatering

Dewatering level as a function of flow rate is calculated by Eq. 4 [18, 19].

$$\%D = 100 - \frac{100(WCW - DCW/dw_r)}{WCW} \quad (4)$$

where WCW is the weight of wet cell cake and DCW is the weight of dry cells. dw_r is the ratio of dry cell weight to wet cell weight after maximum removal of water in extracellular space using filtration.

$$dw_r = \frac{DCW_f}{WCW_f} \quad (5)$$

where DCW_f is the weight of dry cells and WCW_f is the weight of wet cells after filtration.

4. Result and discussion

4.1. Cell growth and product expression

Sorbitol/methanol (1:1, C-mol/C-mol) mixture was determined as a mixed induction strategy based on a previous study [22]. It was shown that the mixed induction strategy effectively induced production and also reduced protease

205 release. In this study, feeding regimen of methanol or mixture was set at a
206 constant rate of 270 mmol carbon•L^{-1}•h^{-1} as recommended by Invitrogen [8].
207 Representative cultivation profiles of methanol and mixed induction were shown
208 in Fig.2 and the key attributes of fermentations were summarized in Table.1. Dry
209 cell weight was around 50 g•L^{-1} prior to induction and reached 132.2 g•L^{-1} and
210 149.1 g•L^{-1} after 96 hours of methanol and mixed induction, respectively. With
211 mixed induction, the biomass was higher because sorbitol metabolism generated
212 more ATP and thus more carbon could be used for biomass synthesis [23].
213 Compared to methanol induction, the mixed induction reduced average oxygen
214 consumption rates (OUR) by 39% from 241.4±21.3 mmol•L^{-1}•h^{-1} to 145.5±6.7
215 mmol•L^{-1}•h^{-1}. Cell viability in the mixed induction was higher (97% versus 93%),
216 which is in agreement with a previous report [11]. Lower product titre and
217 specific productivity were observed after 96 hours of mixed induction. At the
218 harvest time, volumetric yields reached 1.65 g•L^{-1} and 1.12 g•L^{-1}, respectively.
219 One explanation is that reducing methanol concentration decreases *pAOX1*
220 induction and results in a lower productivity. Another possibility is that the
221 impact of sorbitol/methanol dual carbon induction on productivity is cell line
222 specific and cannot be established a priori [12, 24-26]. Only a few protein bands
223 were visualized on the SDS-PAGE gel in both methanol and mixed induction
224 (Fig.3), which indicated that most cells stayed intact even after loss of viability.

225 **4.2. Cell culture characteristics and dewatering efficiencies**

226 Particle size distributions of the cultures from methanol or mixed induction are
227 shown in Fig.4. The cell size distribution did not change during fermentation
228 when pure methanol was used as the inducer, whereas diameter of the cells
229 induced by sorbitol/methanol mixture shifted to smaller values during the
230 induction. D_{50} of the cells from mixed induction decreased from 3.85±0.3 μm to
231 3.14±0.2 μm after 72 hours of induction. It was reported that *P. pastoris* grown
232 on methanol has larger diameter than that on glucose [27], but the comparison of
233 cell culture on methanol and sorbitol has not been reported.

234 Diameter of yeast cells has been found to affect dewatering efficiency in
235 centrifuges [14]. Larger particles are more difficult to be packed in centrifuge and
236 more liquid accumulates in interstitial space [28]. Here dewatering efficiencies
237 of the methanol and mixture induced cell cultures were evaluated using a scale-
238 down model of CSA-1 centrifuge and BTPX305 centrifuge [19] . Compared to
239 the cell culture from methanol induction, the culture from mixed induction had
240 higher dewatering efficiencies in both type of centrifuge (Fig.5). In the range of
241 predicted flow rates, the average dewatering levels improved from 77.3±4.6% to
242 83.0±3.8% ($p<0.01$) in CSA centrifuge and from 78.5±3.6% to 83.1±1.9%
243 ($p<0.01$) in BTPX305 centrifuge. This leads to a prediction of a loss of 41.3±5.3
244 g product from a 1000 L culture induced by methanol, whereas a loss of 17.1±2.1
245 g if mixed induction is used. This indicates that changing induction method is an

effective way to minimize product loss in centrifugal separation. It becomes a valuable process optimization tool specially when high value products are manufactured.

4.3. Identification of host cell proteins

The culture medium after 96 hours of induction was analysed for protein type using HPLC-MS/MS. Overall, a total number of 72 proteins was identified from the mixture induced culture, and the number increased to 96 in the culture with methanol induction. Compared to the mixed induction, more identified proteins localized in cytoplasm and nucleus in the culture from methanol induction (Fig. 6). This indicates that a higher proportion of cell broken although it was not obvious by employing SDS gel assay (Fig.3). More types of proteases were identified in the sample from methanol induction (3 versus 1), which indicates that using methanol induction is likely to cause more proteolytic degradation when products are sensitive to proteases.

In order to show the potential impact of induction on chromatographic steps, distributions of molecular weight (MW) and isoelectric point (PI) of these proteins were compared (Fig.7). In the MW range of 0~24 kDa and PI range of 8.0~14.0, the number of HCPs was much smaller in mixed induction. This indicates that using mixed induction can simplify the purification of some products such as aprotinin (MW/PI, 6.5 kDa, 10.5), Interferon gamma (MW/PI,

18.0 kDa/8.72), Interferon beta (MW/PI, 22.0 kDa/ 9.69) and Keratinocyte growth factor (MW/PI, 22.5 kDa/9.29).

5. Conclusion

In this article, sorbitol/methanol mixed induction was shown to affect both upstream and downstream of *P. pastoris* culture processing. It was found to benefit fermentation by reducing oxygen consumption rate and enhancing cell viability. An ultra scale-down approach enabled the prediction of dewatering levels in the pilot and industrial scale centrifuges. The mixed induction enhanced dewatering and decreased product loss by influencing cell diameter during induction. The mixed induction also benefited the process by improving the product purity and reducing protease release. In summary, sorbitol/methanol mixed induction is an efficient approach to reduce oxygen consumption, minimize product loss by improving dewatering and enhance product quality.

Declare of Interests

The authors have no conflict of interest to declare.

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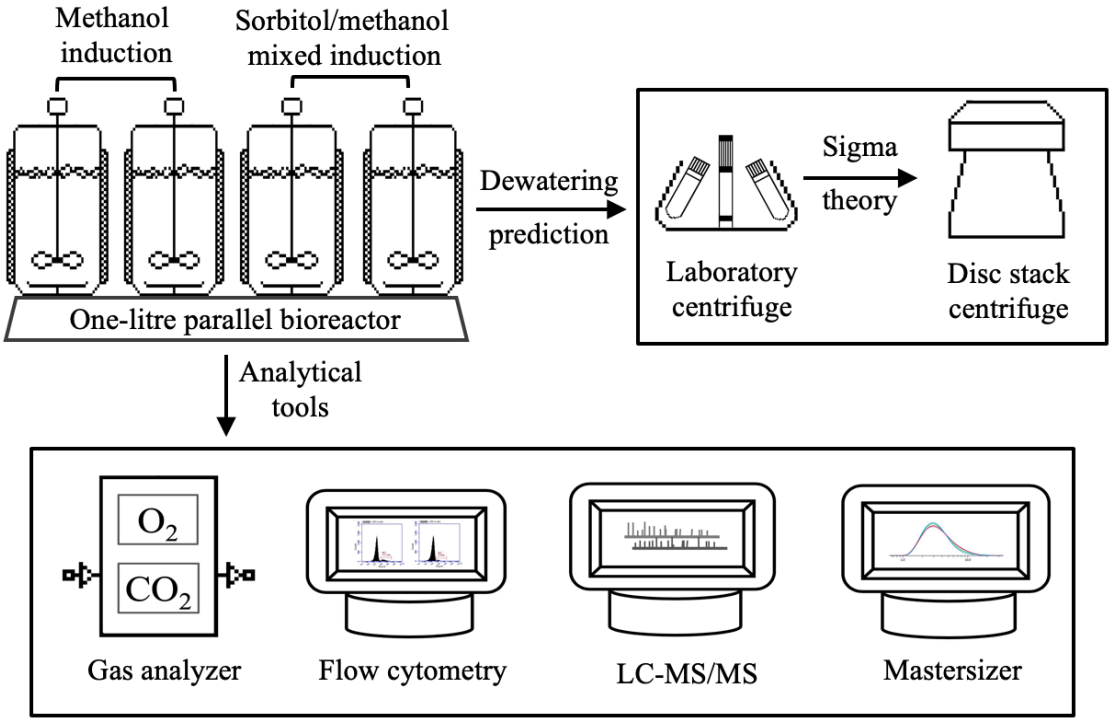
Figures



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391 Fig. 1. Schematic diagram of the major experiments performed in this study.

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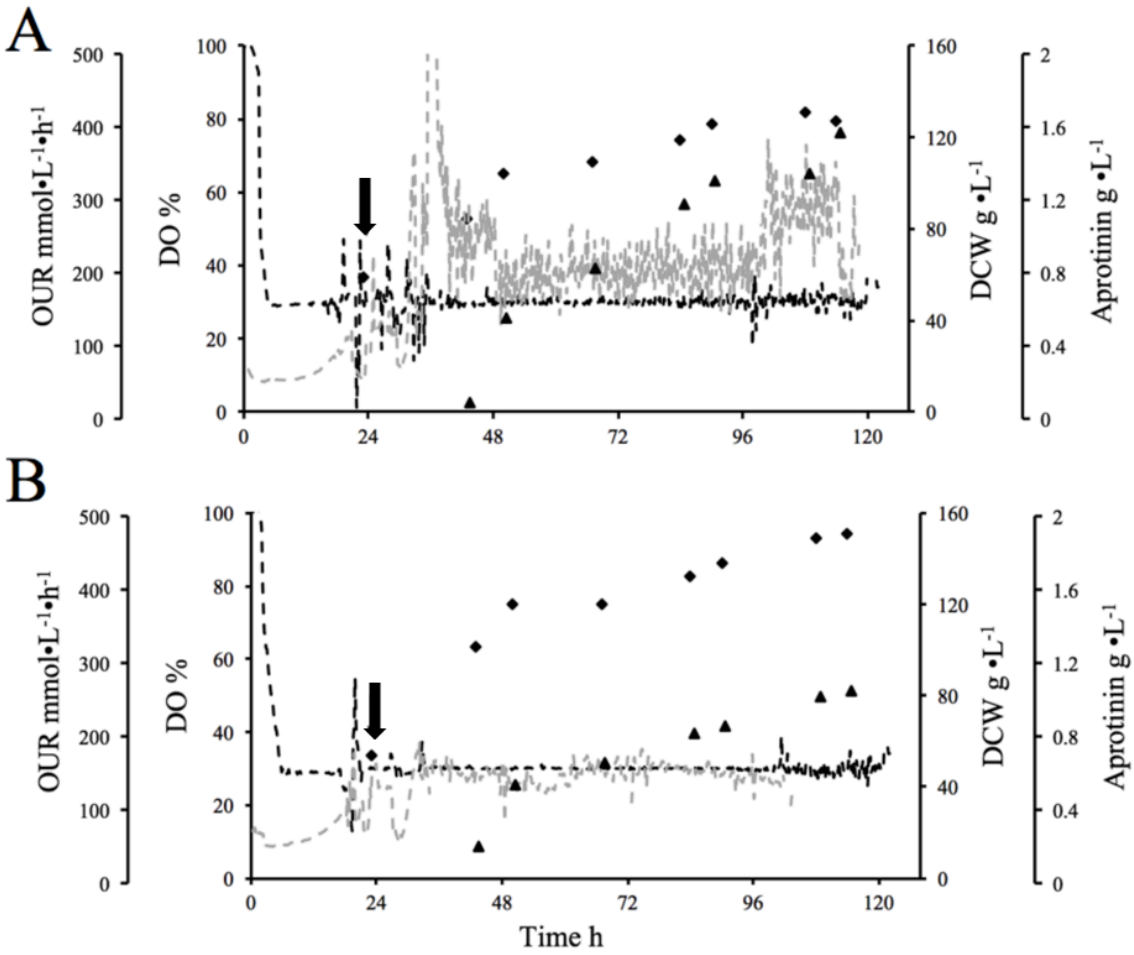

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394 Fig.2 Cultivation profile of *P. pastoris* induced by pure methanol (A) and

395 sorbitol/methanol (1:1, C-mol/C-mol) mixture (B). ◆ dry cell weight (DCW) in

396 g•L^{-1}, ▲ aprotinin concentration in g•L^{-1}, --- dissolved oxygen level in medium,

397 --- oxygen uptake rate of cells in mmol•L^{-1}•h^{-1}, ↓ induction time.

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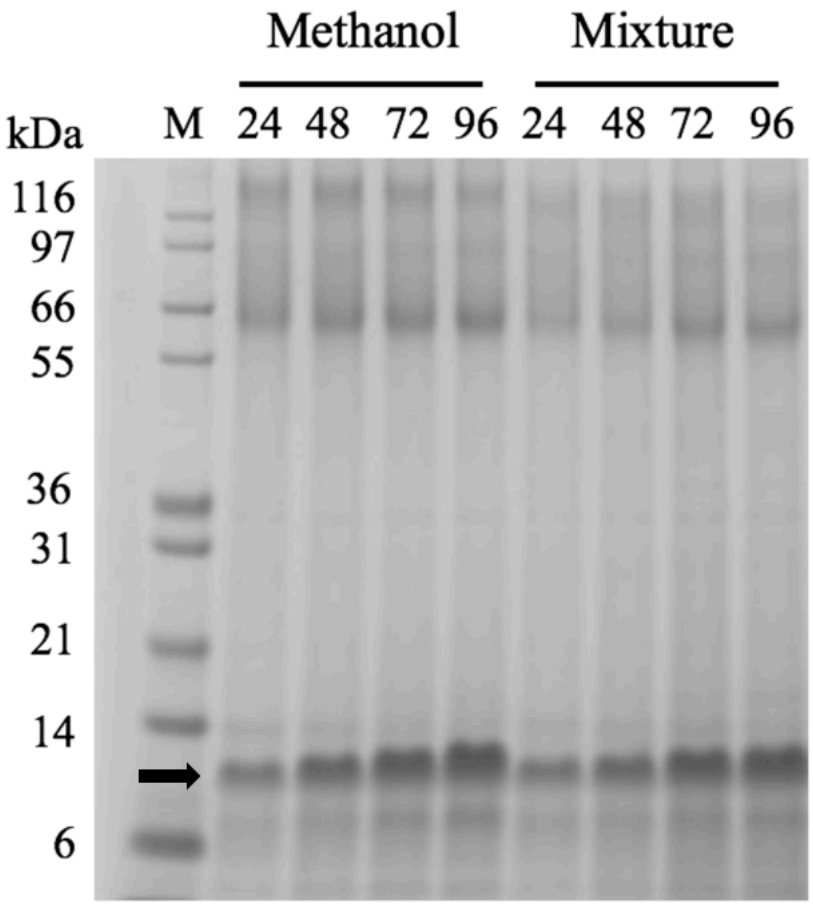

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400 Fig.3 SDS-PAGE analysis of soluble proteins in cell cultures with methanol and

401 mixed induction. Supernatant obtained from 24, 48, 72 and 96 hours of induction

402 was analysed and aprotinin was indicated by the arrow (→).

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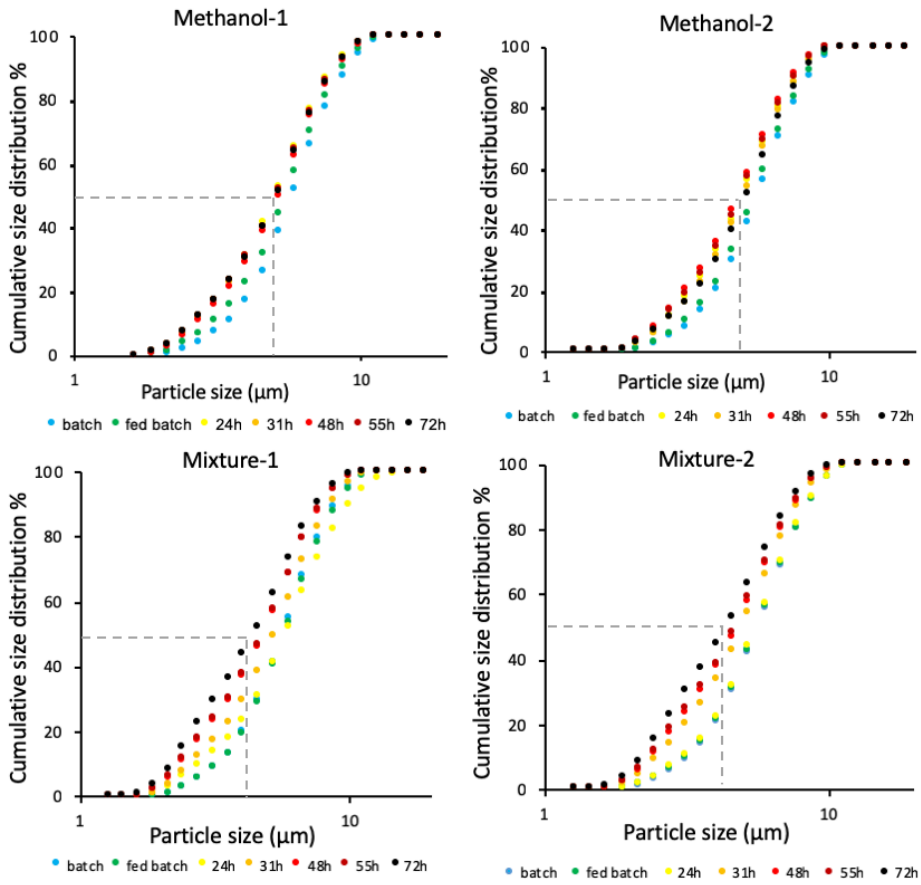

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405 Fig.4 Cumulative cell size distribution at different time points of two repeat
406 fermentations. D_{50} value of culture at harvest time was indicated by the dashed
407 line (---). Samples were collected in batch phase, fed-batch phase and after 24,
408 31, 48, 55 and 72 hours of induction.

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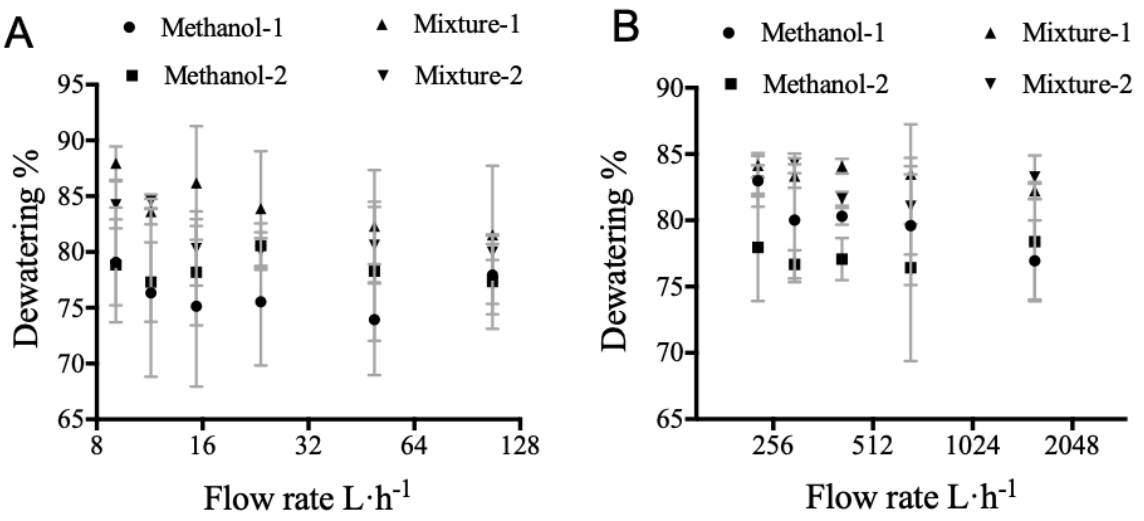

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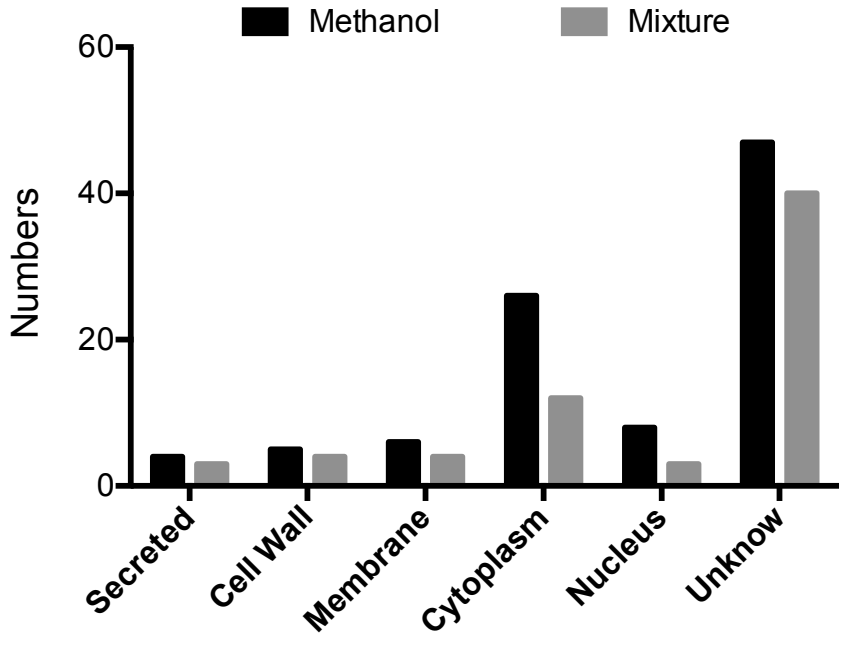
411 Fig.5 Dewatering efficiency of cell cultures from two repeat fermentation
412 induced by methanol or sorbitol/methanol mixture. Dewatering in two centrifuges
413 CSA-1 (A) and BTPX-305 (B) was predicted by scale-down methodology. Data
414 in the graph are presented as mean ± SD (n = 3).



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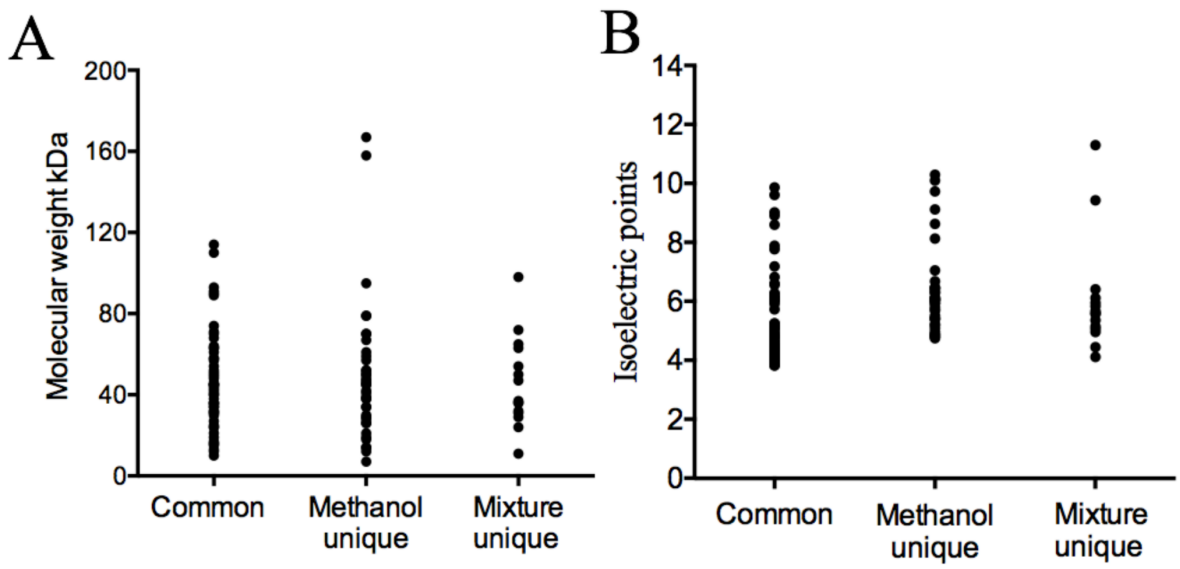
416 Fig.6 Localization of host proteins identified from cell culture induced by
417 methanol and sorbitol/methanol (1:1, C-mol/C-mol) mixture.



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419 Fig.7 Distribution of molecular weights (A) and isoelectric points (B) of HCPs

420 from two induction samples.

421

Tables

	Methanol-1	Methanol-2	Mixture-1	Mixture-2
DCW $g \cdot L^{-1}$	132.8	132.3	152.4	145.7
Viability %	92.5	93.2	97.6	97.8
Aprotinin $g \cdot L^{-1}$	1.68	1.62	1.05	1.18
Biomass yield g DCW$\cdot mol^{-1}$ C	6.19	6.06	8.05	7.80
Specific productivity $mg \cdot g^{-1}$DCW$\cdot h^{-1}$	0.132	0.128	0.072	0.084
OUR $mmol \cdot L^{-1} \cdot h^{-1}$	256.4	226.3	150.2	140.7

Table.1 A summary of biomass, cellular viability, aprotinin concentration, biomass yield, specific productivity and oxygen consumption rate (OUR) in methanol and sorbitol/methanol mixed induction. DCW, viability and aprotinin concentration were measured after 96h' induction. OUR was calculated by averaging OUR values during induction.

Centrifuge	Dimensions	N ($r \cdot s^{-1}$)	C	Σ (m^2)
Eppendorf 5810R	R1 (0.075m) R2 (0.1m)	149	1.0	0.66~0.77
Beckman Coulter Avanti J-E	R1 (0.073m) R2 (0.102m)	92	1.0	1.12~1.82
CSA-1	R1 (0.026m) R2 (0.055m) n (45) θ (38.5°)	162	0.4	1444
BTPX-305	R1 (0.036m) R2 (0.085m) n (82) θ (40°)	125	0.4	7127

429 Table 2. Dimensions of the used laboratory and industrial scale centrifuges.

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