J Ind Microbiol Biotechnol (2010) 37:323–333
DOI 10.1007/s10295-009-0677-x

ORIGINAL PAPER

Improved production of polygalacturonate lyase by combining a pH and online methanol control strategy in a two-stage induction phase with a shift in the transition phase

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Received: 23 March 2009 / Accepted: 30 November 2009 / Published online: 20 December 2009
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Abstract Polygalacturonate lyase is a kind of enzyme that is abundantly used in the textile industry for cotton scouring. Previously, we reconstructed the polygalacturonate lyase gene in *Pichia pastoris* for the expression of this enzyme. To enhance the production of polygalacturonate lyase (PGL), a combined strategy was formulated by combining online methanol control and two-stage pH control strategies. For the two-stage pH control strategy during the growth phase, the pH was controlled at 5.5, and in the induction phase different pH levels were investigated for the optimum enzyme production. During the online methanol control strategy, the different levels of methanol (v/v) were investigated for the best enzyme production at pH 5.5. These two strategies were combined together for enhanced PGL productivity, and the induction phase was divided into two stages in which methanol concentrations were maintained at different levels online. The transition phase was introduced during the induction phase instead of introducing it after the growth phase. The two-stage combination strategy was formulated on the bases of methanol consumption of cells, optimal pH, cell viability and the production of polygalacturonate lyase by *P. pastoris*. By using this strategy, the production was doubled compared with common conditions, and the highest polygalacturonate lyase activity reached 1,631 U/ml. This strategy proved to be very useful for the enhancement of polygalacturonate lyase production by achieving higher cell viability, alcohol oxidase activity and phosphate-related compounds of the cells during the induction phase.

Keywords *Pichia pastoris* · Polygalacturonate lyase (PGL) · Two-stage induction phase · pH control strategy · Online methanol control strategy

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Introduction

Pectinase (also known as polygalacturonase) is the collective term for a row of enzymes that are able to break down pectin [1]. Pectinases are both alkaline and acidic, and are often used in combination with other enzymes foremost in the preparation of fruit and vegetable juices to increase the yield. The polygalacturonases (PGase) involved in the hydrolysis of pectic substances are of two types, endo and exo. It is mainly extra-cellular endo-PGase that breaks down the pectate by hydrolysis of α-1,4-glycosidic linkages in a random way [1]. Endo-PGases are found in fungi, bacteria and many yeasts [2]. They are also found in parasitic nematodes [3] and have been reported in many microorganisms, including *Aureobasidium pullulans*, *Rhizoctonia solani Kuhn* [4], *Fusarium moniliforme* [5], *Neurospora crassa* [6], *Rhizopus stolonifer* [7], *Aspergillus* sp. [8], *Thermomyces lanuginosus* [9] and *Peacilomyces clavisporus* [10]. Exo-PGases are of two types: fungal exo-PGases, which produce monogalacturonic acid, and the bacterial exo-PGases, which produce digalacturonic acid. Polygalacturonate lyase (PGL) is produced mainly by bacteria belonging to *Bacillus, Erwinia, Pseudomonas* and *Streptomyces*, and in a few fungi causing food spoilage.

Very few reports are available on PGL from protozoa. Endo-PGL activity was observed in cell free extracts of rumen ciliate protozoa, viz., *Ostracodinium obtusum* and *Entodinium caudatum* [11]. A human anaerobic intestinal bacterium, *Clostridium butyricum-beijerinckii* [12], produced 0.21 U/ml of endo PGL and 0.11 U/ml of exo PGL when pectic acid was used as a carbon source. The presence of a complex nitrogen source such as peptone in medium enhanced the production of PGL in *Bacillus* sp. [13]. *Pseudomonas marginalis* [14] produced both pectate lyase (PL) and PGL when grown in enriched medium containing 0.1% citrus pectin. Psychrophiles and thermophiles produce PGL. *Streptomyces thermovulgaris* CR42 produced PGL at pH 7.6 and a temperature of 55°C [15–18]. A thermophilic *Bacillus licheniformis* [19] isolated from a sugar factory residue produced extracellular PGL. *Chryseomonas leteola* produced three isozymes, PGL I, PGL II and PGL III [17]. The optimum temperature for PGL production was 20–24°C, and production of PGL I and PGL III were regulated by temperature. The effect of aeration and agitation on fungal morphology is crucial for PL production. PL production by *A. niger* was also enhanced by 4.6-fold when the concentration of sugars in the medium increased from 1.5 to 15% (w/v) [20]. Concomitant production of PL and citric acid by *A. niger* was studied in a stirrer tank reactor (STR). At optimal conditions of aeration and agitation, the maximal PL activity was 0.5 U/ml when sucrose (14%) and dry whey (1%) were used as the carbon source [21].

Our research was based on the strategic planning and changing of the existing fermentation strategies to improve the polygalacturonic acid lyase productivity by *P. pastoris*. Different feeding strategies have been applied to enhance the biomass [22] as it is considered the base of better productivity, and it has been observed that accumulation of a non-fermentable carbon source with methanol does not affect the level of expression of recombinant protein [23]. One of the reasons for using *P. pastoris* in our study was its ability to grow at higher methanol concentrations (up to 20 g/l), wider pH range and production of fewer oxidized toxic metabolites from methanol oxidation as compared to *Hansenula polymorpha*. There are two phenotypes of *P. pastoris* (Mut^s and Mut^+), between them the Mut^+ strain is more sensitive to overdose of methanol [24, 25]; hence, a much stricter control of methanol feed is needed for the bioreaction. Enhanced protein expression can be achieved by three major factors: achievement of high cell density cultures [26], efficient expression by controlling the methanol at an adequate concentration range and utilization of an inexpensive medium to reduce the production cost. The enhanced polygalacturonic acid lyase production by *P. pastoris* is basically divided into two phases: the fed batch growth phase and the induction phase. During the fed batch phase, the yeast utilizes glycerol, and biomass is accumulated. A third phase or transition phase is introduced before the methanol induction in order to repress the alcohol oxidase (AOX) gene for better production. In the induction phase, methanol alone or in combination with lower concentrations of glycerol (mixed feed) is added, and the biosynthesis of the recombinant protein is initiated [27]. The yeast is sensitive to methanol; hence, the level of methanol has to be maintained at below 0.4%. Methanol feeding strategies can be metabolism related based on parameters such as methanol consumption [28, 29], oxygen consumption [30], CO_2 or pH related. In our work the online methanol feeding (induction phase) strategy was combined with pH and bifurcated in two phases on the basis of time and concentration. The pH level was varied in the growth phase and induction phase; the transition phase was introduced between the growth phase and the induction phase. In the first period of induction, a lower level of methanol concentration was selected based on cell viability and dry cell weight (DCW), and in the second period it was increased to higher level for optimum PGL production. A short transition was introduced during which glycerol was provided at a limiting rate for 1 h. The shift in the transition phase has never been reported before, as the transition is provided only after the growth phase both in offline and online methanol induction strategies [31]. A better understanding can be achieved of how methanol concentrations can be manipulated combined with pH to enhance the extracellular recombinant protein production with a cost-effective industrial-scale method in *P. pastoris* GS115.

Materials and methods

The strain used in this study was a recombinant *P. Pastoris* GS115, with an alkaline polygalacturonic acid lyase gene integrated from the *Bacillus* sp. via vector pPIC9K [32].

Two media, seed culture medium (SCM) and basal salt medium (BSM), were used. SCM was prepared in 500-ml flasks and contained per liter: glucose 20 g, peptone 20 g and yeast extract 10 g. About 800 µl of seed stock stored at −80°C was inoculated into flasks containing 30% SCM pre-sterilized at 121°C for 10 min. The seed was incubated at 30°C for 24 h at 200 rpm. The BSM contained glycerol 40 g/l, K_2SO_4 18 g/l, $MgSO_4 \cdot 7H_2O$ 14.9 g/l, KOH 4.13 g/l, H_3PO_4 27 ml/l, $CaSO_4$ 0.93 g/l and 4.4 ml/l of trace metal solution (TMS). TMS contained: $CuSO_4 \cdot 5H_2O$ 6 g/l, KI 0.08 g/l, $MnSO_4 \cdot H_2O$ 3 g/l, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g/l, H_3BO_3 0.2 g/l, $CoCl_2$ 0.5 g/l, ZnCl 20 g/l, $FeSO_4 \cdot 7H_2O$ 65 g/l, biotin 0.2 g/l and H_2SO_4 5 ml. The BSM was made in a pre-sterilized 3-l fermentor with a working volume of 1.2 l. The bioreactor (Biotron Intelligent Fermentor, Korea) with the BSM was sterilized at 115°C for 15 min

with post addition of filter sterilized TMS. Dissolved oxygen (DO) was adjusted above 10% by cascade control agitation rate between 500 and 950 rpm, and sterilized air was pumped in at the rate of 5 l/min. The pH was automatically controlled at 5.5 by adding 25% ammonium solution.

Fermentation conditions

Glycerol solution at the concentration of 50% (w/v) was provided in the fermentation medium containing trace metal solution at the concentration of 12 ml/l and sterilized at 115°C for 10 min. The cells were allowed to grow in the medium (growth phase), and it took 12–16 h before the level of DO started to rise. With the increase in DO tension, the glycerol fed batch phase was initiated. To maintain glycerol concentration under a certain level in the glycerol fed batch phase, glycerol was fed using an exponential feeding mode [33]. The fed batch phase ended when the glycerol was exhausted, which was indicated by a sudden increase in DO (so-called DO spike). The cell density (cell biomass per liter) at the end of the batch phase (Xgb) was obtained by centrifugation (Hitachi, HimacCR22G II Japan) at 2,000×*g* (1 g/l of wet cell weight ≈ 0.27 g dry cells/l ≈ 1 OD_{600}). The specific growth rate (i) is 0.177 h^{-1} in fed batch phase when there is no limiting factor on growth rate [34]. By taking a sample at some point (*t*s) during the fed batch phase and measuring the cell density (*X*s), the end time of batch phase, t_{gb}, can be predicted by the following Eq. 1.

$$t_{gb} = t_s + 1/\mu_{gb}\ln(Xgb/Xs) = ts + 1/0.177\text{In}(105/Xs) \quad (1)$$

In the induction phase the carbon source was methanol containing 12 ml/l TMS. Any change in pH was controlled with the addition of 35% H_3PO_4 and 25% ammonium solution; the samples were taken after every 6 h.

Two-stage pH control strategy

For the pH control strategy the pH during the growth phase was maintained at pH 5.5, which is the most suitable pH for the growth of *P. pastoris* GS115. The objective of keeping the pH at this level during the growth phase was to enable high cell density cultures for better PGL productivity. After the growth phase ended, which was indicated by a DO spike, a transition phase was introduced for the AOX1 gene to be repressed for the induction phase [33]. This transition phase is important as the yeast is sensitive to methanol. After a 3-h transition, the methanol induction was commenced, during which various pH levels were investigated for the enhanced PGL productivity.

Online methanol feed during the induction phase

For the online methanol induction phase, different methanol feeds (v/v) to enhance the production at a constant pH of 5.5 in the induction phase were investigated. The methanol levels investigated were 8, 12, 16, 20 and 24 ml/l. The induction phase lasted for 100 h. The level of methanol was kept constant throughout the induction phase to avoid any excess of the carbon source, which could lead to cell death. A methanol probe (Super Info Tech. Co. Ltd., Shanghai, China) was used to monitor the methanol level in the bioreactor.

Combination of pH and methanol feed strategy

This strategy was a combination of the two-stage pH strategy and online methanol feed strategy. In the combined strategy, the induction phase was divided in two stages; in each stage the residual methanol level (v/v) was different. Using this strategy, the induction phase was immediately initiated after the glycerol phase without the transition phase. This transition phase was introduced after the first stage of induction (40 h), during which methanol was provided at a low level (12 ml/l); the transition phase lasted for 2 h, during which glycerol (50% w/v) was given for 1 h at the rate of 5 ml/h. After 2 h, a DO spike occurred, and the next stage of induction was initiated, during which the methanol concentration was kept at higher level (16 ml/l).

Analytical methods

For the determination of the dry cell mass, a 10-ml sample was taken every 6 h during the induction phase. The samples were placed in pre-weighed sampling tubes, and the OD was determined at 600 nm with a spectrophotometer. The samples were centrifuged at 5,000×*g* at 4°C for 10 min; the supernatant was separated from the biomass for further analysis and stored at −20°C. The biomass was initially dried at 80°C for 24 h and then further dried at 100°C for another 24 h before weighing. The relationship of the OD and the dry cell weight is calculated by the following Eq. 2 [34].

$$\text{Drycell weight (in g/L)} = 0.22 \times OD_{660} \quad (2)$$

The polygalacturonic acid lyase activity was determined by the method described by Wang et al. [33]. The activity was based on the measurement of the absorbance cleavage of a 1, 4-glycoside linkage at the non-reducing terminal of galacturonic acid, forming unsaturated bonds between C_4 and C_5. The reaction mixture contained 2 ml of 0.2% (w/v) polygalacturonic acid (Sigma Chemical Co., type P7276)

in 200 mM glycine-NaOH buffer (pH 9.4, 50 mM glycine, 17.5 mM NaOH and 0.44 mM $CaCl_2$) and 20 ml of diluted enzyme. Reaction mixtures were incubated at 45°C for 15 min, and the reaction was terminated by the addition of 3 ml of 300 mM phosphoric acid (H_3PO_4). The activity was determined by a spectrophotometer (Shimadzu, UV-2450-PC) at 235 nm and room temperature. One unit of enzyme activity is defined as the formation of one mole of unsaturated polygalacturonic acid per minute with a molar extinction coefficient of 4,600.

The cell viability was determined by methylene blue dye exclusion technique according to the procedure described by Beney [35]. The bioreaction samples taken at regular intervals and suitably diluted (approximately $OD_{600} = 20$–30; OD is the optical density) were mixed with an equal volume of methylene blue dye solution for 1 min and then mounted on a hemo-cytometer to count the percentage of live cells in the total population. The cells that took up the dye had a deeper blue color and were considered dead; the cells that did not take up the dye appeared translucent and were considered alive.

The protease was determined by the Quanti Cleave protease assay kit (Pierce, USA). The assay method uses succinylated casein and trinitrobenzensulfonic acid (TNBSA). Succinylated casein is a native casein that has been treated with succinic anhydride to block primary amines on the surface of the protein. In the presence of protease, the succinylated casein is cleaved at peptide bonds, thereby exposing primary amines (predominantly alpha amines). TNBSA reacts with these exposed primary amines to produce an orange-yellow product whose intensity may be measured at 450 nm. The increase in the color relative to the sample without succinylated casein is a measure of protease activity in the sample. The change in absorbance at 450 nm is determined by subtracting the absorbance of the blank from the corresponding casein well. The change in absorbance is generated by the proteolytic activity of the protease [36].

Adenosine diphosphate (ADP), adenosine triphosphate (ATP) and adenosine monophosphate (AMP) were determined with the methods as follows. Samples taken at regular time intervals were placed in liquid nitrogen prior to freezing at −80°C to terminate the intracellular activity. The samples were thawed on ice, and 10 ml was pipetted directly into an equal volume of fresh pre-cooled (4°C) 0.5 mM $HClO_4$ containing 1 mM ethylene diamine tetra-acetic acid (EDTA), mixed vigorously and extracted for 30 min at 4°C with periodic agitation. Cell debris was discarded, and the supernatant was stored at −20°C for further analysis. The concentrations of ADP, ATP and AMP were determined by high pressure liquid chromatography (HPLC) (Agilent 1100) with a hypersil ODS column (4.6 × 200 nm) packed with C18 packing material, 5 μm particle size and a 254-nm UV detector. Solvent A was CH_3OH and solvent B was 0.1 mM KH_2PO_4 at pH 7.0, adjusted with 0.1 mM KOH solution. The gradient program used was 100% of solvent B from 0 to 5 min with a flow rate of 1 ml/min, and 90% of solvent B from 5 to 25 min with a flow rate of 1.5 ml/min [37]. Energy charge (EC) was calculated as follows:

$$\mathrm{EC} = \mathrm{ATP} + 0.5\,\mathrm{ADP}/\acute{\mathrm{O}}\,\mathrm{NT}, \text{ where}$$

$$\acute{\mathrm{O}}\,\mathrm{NT} = \mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP}.$$

Alcohol oxidase activity was determined as follows. At regular intervals, 10 ml of samples was taken during the induction phase and placed in liquid nitrogen to stop all metabolic activities. The samples were thawed, and the cells were harvested by centrifuging at 10,000×*g* for 10 min. The cells were washed twice with 50 mM phosphate buffer (pH 7.0). The cells were re-suspended in the same buffer. About 1 ml of the sample was taken and disrupted by sonication at a vibrational frequency of 50 hertz (Hz) for 10 min at 0°C. Cell debris was collected by centrifugation at 10,000×*g* for 20 min, and the supernatant was used as cell-free extract. The alcohol oxidase activity was assayed by measuring the H_2O_2 produced during oxidation of methanol [38]. The standard assay mixture contained 100 μmol phosphate buffer (pH 7.0), 1 μmol 4-aminoantipyrine, 4.3 μmol phenol, 10 units of peroxidase, 200 μmol of methanol and enzyme in a total volume of 3 ml. The reaction was done at 37°C for 10 min, and the production of H_2O_2 was measured by the appearance of the quinoneimine dye formed by the coupling with 4-aminoantipyrine and phenol. One enzyme unit is defined as formation of 1 μmol of H_2O_2 per min under these conditions.

Statistical analysis

All the values obtained were the mean of three values, and the standard deviation was calculated for each value.

Results and discussion

Impact of two-stage pH control strategy on polygalacturonate lyase production

To investigate the effect of pH on PGL production, different parameters were studied at different pH levels of 3.5, 4.0, 4.5, 5.0, 5.5 and 6.5. Dry cell weight (DCW) at pH 4.5 reached the maximum of 150 g/l during the 75–85 h (Fig. 1a). It was observed that dry cell weight on an average remained at 125 g/l when the yeast was grown at pH 3.5, 4.0, 5.0, 5.5 (control) and 6.5, and maximum

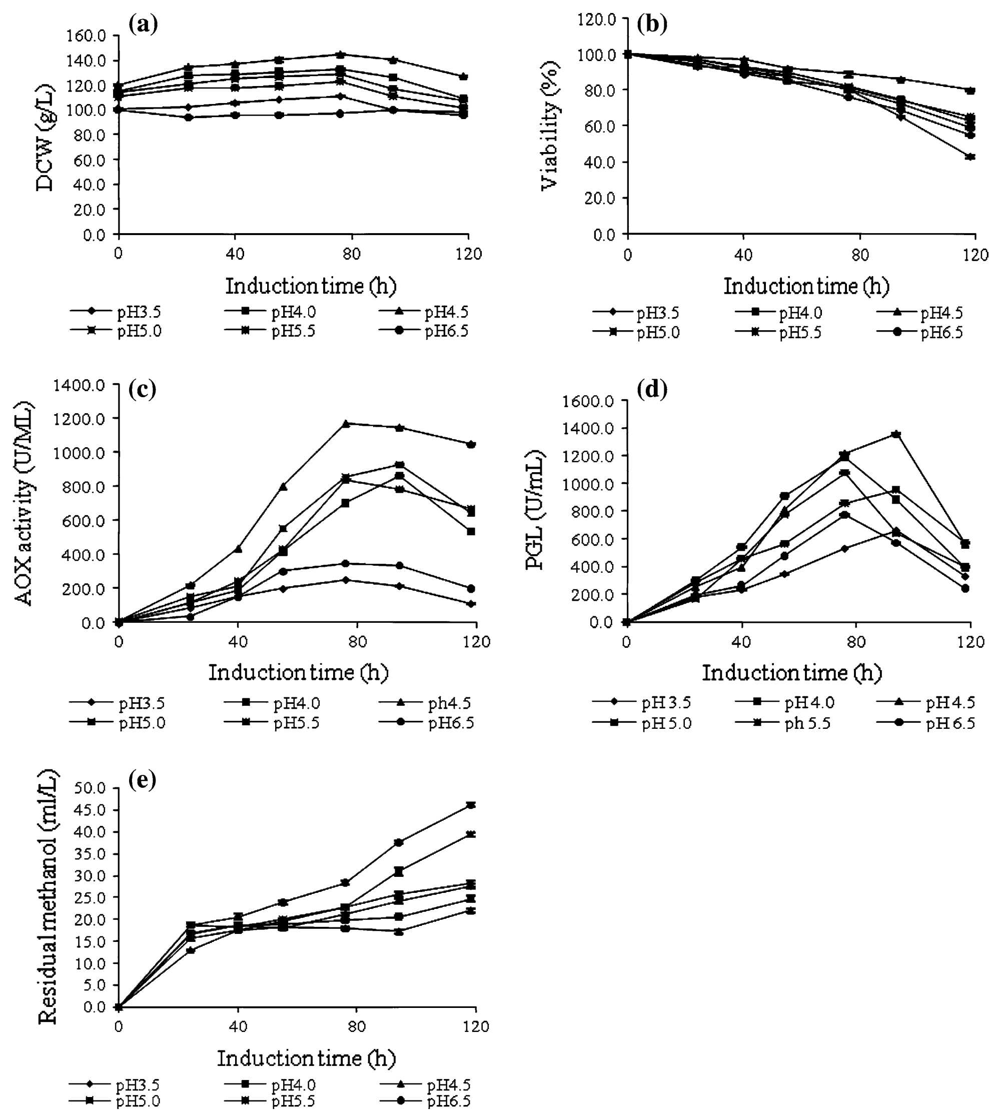

Fig. 1 Impact of two-stage pH control strategy on DCW (**a**), cell viability (**b**), AOX activity (**c**), PGL activity (**d**) and residual methanol (**e**) during the induction phase. pH 5.5 was the control; $n = 3$

DCWs after 80 h were 110.7, 132.3, 128.4, 122.6 and 100.4 g/l, respectively. As determined by the methylene blue dye exclusion technique, the cell viability remained above 80% even by the end of the fermentation for pH 4.5. The cell viability was higher with pH 4.5 as compared to other pH levels (Fig. 1b). The AOX activity achieved by the two-stage pH control strategy (Fig. 1c) shows that the maximum AOX activity was found to be pH 4.5 at 1,170 U/ml and 7.8 U/g of DCW. The lowest activity of AOX was achieved with pH 3.5 at 250 U/ml and 2.2 U/g of DCW, and with pH 6.5 at 311.5 U/ml and 3.3 U/g of DCW.

It was observed that the protease activity increased gradually as the induction phase proceeded. If the pH of the fermentation broth is not controlled, it continues decreasing [39, 40] and hence causing cell death. Under normal circumstances, if the pH is allowed to drop from 5.0 to 3.0 in the growth phase, the proteolytic degradation can be reduced [40], but at pH 2.0 cell activity decreased as a result of cell death and lysis. During the initial 40 h of induction, the protease activity for pH 3.5, 4.0, 5.0, 5.5 and 6.5 was 0.02 U/ml on average, but with pH 4.5 it was 0.002 U/ml. By the end of the induction phase, the protease activity at various pH levels had increased to 0.3, 0.3, 0.25, 0.28 and 0.23 U/ml, respectively, except at pH 4.5 at 0.15 U/ml, which was the least among all (results not shown). At pH 3.5 and 4.0, the protease activity was higher than pH 4.5 since at lower pH levels the cell growth ceased and cell activity decreased. At pH 3.5 and 4.0 the cell viability was also found to be low, which could be the result of higher protease activity. All these parameters were investigated for a higher PGL productivity at an appropriate pH level. In the control (pH 5.5), the PGL activity reached a maximum of 956.5 U/ml by the end of 100 h. Most of the maximum values at different pH levels were achieved between 80 and 90 h, with pH 3.5, 4.0, 5.0 and

6.5 at 657.9, 1,185.5, 1,075.3 and 773.9 U/ml, respectively. With pH 4.5 a maximum of 1,362.3 U/ml was achieved by the end of 100 h (Fig. 1d).

The methanol consumed by the yeast cells grown at pH 4.5 was higher than at other pH values. In the initial 40 h of induction phase at pH 4.5, the concentration of residual methanol remained below 15 ml/l (Fig. 1e). A static state of 18 ml/l of residual methanol was achieved between 40 and 80 h, and by the end of 100 h of induction it continued rising to 22 ml/l. At other pH values no static state was observed, and the residual methanol continued rising and ended up at high levels of >25 ml/l, with pH 6.5 registering the very high value at the end of the fermentation of 46 ml/l. At pH 5.5 residual methanol accumulations resulted in cell death and lysis, hence causing the protease production as much of the phosphate compounds was lost at high methanol concentration. Hence, this also answered the question: why was pH 5.5 not used at the induction phase when during the growth phase it was the best suitable pH for cell growth of *P. pastoris* as reported by Cregg et al. [41]?

On the basis of the results obtained, it was concluded that in a two-stage pH 4.5 control when the pH is varied from pH 5.5 in the growth phase to pH 4.5 in the induction phase, the PGL produced is much better, which has been proven by also investigating other parameters as discussed above. The next step was to investigate if online methanol could produce remarkable results or not; for this purpose we carried out online methanol control fermentations.

Impact of online methanol control on polygalacturonate lyase production

Different online methanol levels were investigated for enhanced PGL production at a constant pH of 5.5. At different methanol levels of 8, 12, 16, 20 and 24 ml/l, the maximum DCWs achieved were 127.4, 140, 162, 135 and 123 g/l, respectively. During the initial 40 h, the methanol level of 12 ml/l had the maximum DCW of 127 g/l (Fig. 2a) compared to other levels and the control at a constant methanol rate of 10 ml/h. The DCW of the control reached the maximum of 120 g/l and at 40 h was 118 g/l. With online methanol at constant levels of 8, 12, 16, 20 and 24 ml/l, the maximum values of AOX achieved were 501.8, 632.6, 1,097.3, 834.2 and 487.4 U/ml, respectively, in the late 70 h (Fig. 2b). Protease activity with online methanol concentration at 8, 12, 16, 20 and 24 ml/l was 0.29, 0.34, 0.28, 0.3 and 0.31 U/ml, respectively (Fig. 2c). In the initial 40 h of induction, 12 ml/l of methanol showed the least activity of 0.03 U/ml, and in the later 70 h 16 ml/l of methanol showed the least activity of 0.16 U/ml. The cell viability at the end of fermentation was reported to be 74% with 12 ml/l methanol and a minimum of 42% with 24 ml/l, but with 16 ml/l the cell viability was reported to be 77% (Fig. 2d). During the initial 40 h the cell viability for methanol at 12 ml/l was the maximum with 97% followed by 16 ml/l at 95%. With 8 ml/l the cell viability decreased to as low as 59%. The protease activity was low, but due to the unavailability of sufficient methanol in the culture, the cell viability declined gradually up to 59% with a methanol level of 8 ml/l. Assessment of cell viability during *Pichia pastoris* fermentation using flow cytometry showed that almost 35% of the cells become nonviable [42], and dead cells retained the cell proteins as long as they were not lysed.

With the online methanol control strategy with the methanol concentration at 8 ml/l, PGL reached a maximum of 1,102.9 U/ml, and at the end of fermentation PGL was 420.2 U/ml. With a methanol concentration of 12 ml/l, PGL reached the maximum of 114.9 U/ml and was 463.7 U/ml at the end of fermentation. With the methanol concentration of 16 ml/l, PGL reached the maximal value of 1,252.1 U/ml and was 494.2 U/ml at the end of fermentation. With the methanol concentration of 20 ml/l, PGL reached the maximal value of 849.2 U/ml and was 250 U/ml at the end of fermentation. With a methanol concentration of 24 ml/l, PGL reached the maximum of 484.0 U/ml and was 213 U/ml at the end of fermentation (Fig. 2e). The PGL produced was also investigated with the internal phosphate-related compounds of the cell; the maximum ATP, ADP and AMP achieved were 1.2, 0.04 and 0.28 g/l, respectively, with a methanol level of 16 ml/l (results not shown). The minimum values achieved were with methanol level of 8 ml/l and a residual methanol level of 24 ml/l; the ATP level on average was at 0.3 and 0.2 g/l, ADP at 0.01 and 0.01 g/l, and AMP at 0.15 and 0.17 g/l, respectively.

Online methanol control gave a new dimension to our research to improve the PGL productivity. It was observed that when the residual methanol was controlled online, it enhanced the productivity. Although the productivity was lower than the two-stage pH control, it was better than the control. In our next set of fermentations we combined both these strategies and shifted the transition phase after the growth to during the induction. The induction phase was also divided into two stages on the basis of the results obtained with the online methanol control and two-stage pH control strategy.

Effect of combined strategies on polygalacturonate lyase production

In this combination strategy, two-stage pH control and online methanol control strategies were combined in a single strategy. The methanol induction was immediately initiated after the growth phase. After 40 h of the induction

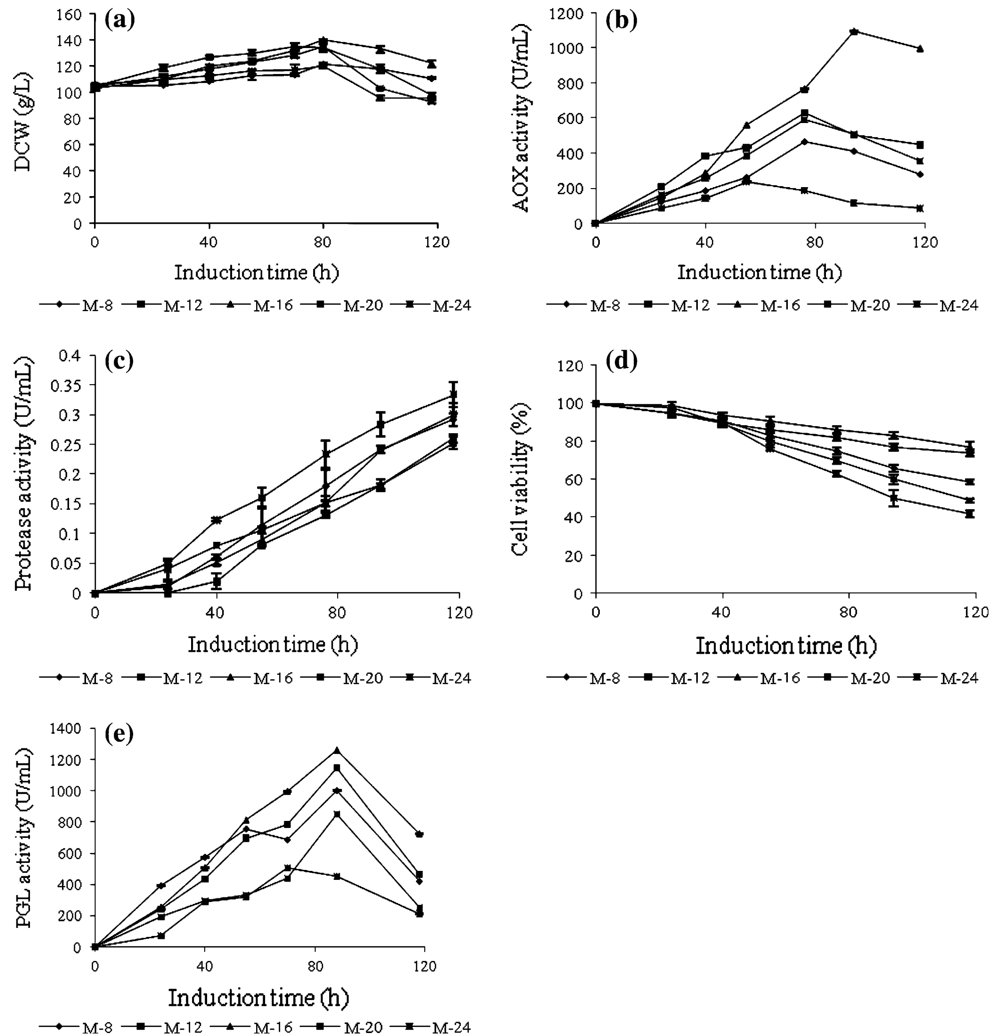

Fig. 2 Impact of online methanol control strategy at constant levels of pH 4.5 on DCW (**a**), AOX activity (**b**), protease activity (**c**), cell viability (**d**) and PGL activity (**e**) during the induction phase; $n = 3$. *M-8* with methanol level of 8 ml/l, *M-12* with methanol level of 12 ml/l, *M-16* with methanol level of 16 ml/l, *M-20* with methanol level of 20 ml/l, *M-24* with methanol level of 24 ml/l

phase, the transition phase was introduced by halting the methanol induction. Glycerol at a limiting rate was introduced into the culture after 20 min, since methanol is completely consumed within 20 min after turning off the supply [43]. It was observed that after the transition phase, the DCW was as high as 140 g/l, and an average of 141 g/l DCW was maintained by the combination of low level of methanol (12 ml/l) with a high level of methanol (16 ml/l) in the second induction stage. This resulted in a DCW as high as 162 g/l (Fig. 3a). With combinations of a low level of methanol (12 ml/l) with 20 and 24 ml/l of methanol, the maximum DCW reached 135 and 123 g/l, with an average of 110 and 121 g/l, respectively. During normal fermentation processes, a continuous supply is provided or a mixed feeding of methanol and glycerol is induced. The combination strategy was different from the traditional fermentation methods because the induction phase itself increased the biomass (Fig. 3a) and enhanced the production of PGL (Fig. 3e). With the traditional strategy, all parameters continuously declined since the energy of the cells was not replenished. One of the reasons for the high biomass accumulation was the transition phase, which reduced the stress on the cells of *P. pastoris* and eliminated the weak cells. The cell viability test in Fig. 3d confirmed that after the transition phase the viability was as high as 100%, which resulted in increased cell biomass. Not only the biomass but also the AOX activity (Fig. 3b) in the second stage of the induction phase was much higher at 1,473 U/ml compared to the results at 16 ml/l of methanol induced constantly in the entire induction phase. On average, the AOX activity remained more than 1,100 U/ml during the second stage of the induction phase. The AOX activity after the transition phase with 16 ml/l of methanol was higher at 385 U/ml compared to the result of 254.7 U/ml with the combination strategy. During the induction phase of the combination strategy, this increased AOX activity was due to higher cell viability. Since a low level of methanol concentration had already been induced after the growth phase, the AOX gene had been repressed. The transition phase with growth-limiting levels of glycerol

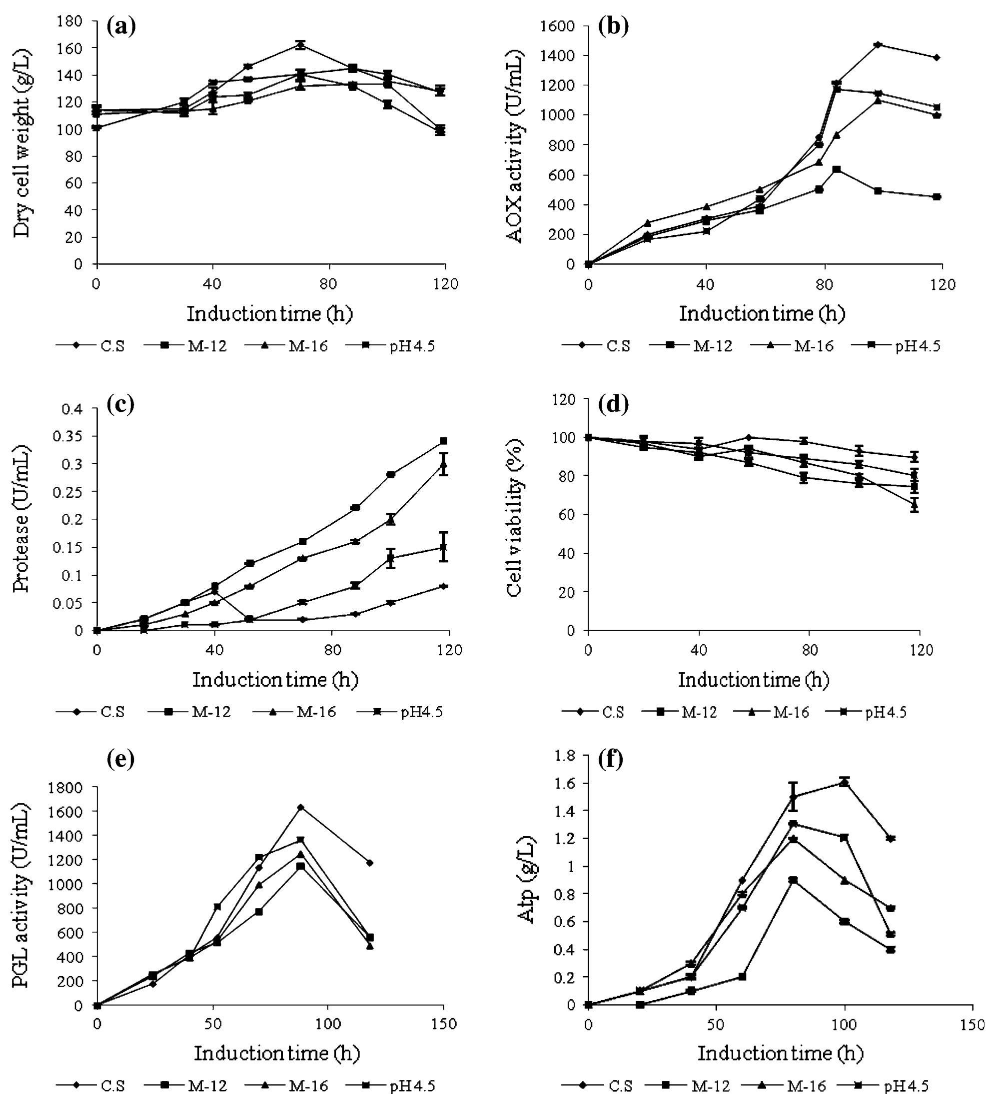

Fig. 3 Effect of combining two-stage pH control and online methanol control strategies on the DCW (**a**), AOX activity (**b**), protease activity (**c**), cell viability (**d**), PGL activity (**e**) and ATP concentration of the cells (**f**) during the induction phase; $n = 3$. *CS* With combined strategies, *M-12* with methanol level of 12 ml/l, *M-16* with methanol level of 16 ml/l

resulted in increased AOX activity, and this in return increased the PGL production. The protease activity from 40 to 90 h of induction phase in the combination strategy remained at an average of 0.075 U/ml. After the transition phase the protease activity was at 0.05 U/ml (Fig. 3c). This protease activity was much lower compared to the two-stage pH control and online methanol control. As discussed earlier, the combination of a lower level of residual methanol with a higher level is beneficial in *P. pastoris* GS115 cultures; it increased the viability and hence reduced the cell death in the later stage of the induction phase. With increased cell viability with higher residual methanol, the dead cells retained the cell structure [42]. Apart from different residual methanol levels, the pH also had a very positive influence on the protease activity. It has been reported that lower pH levels reduce extracellular protease [44], but at pH 2.0 the cell activities ceased. Our novel strategy helped to reduce the proteolytic degradation and avoid cell lysis compared to pH or online methanol induction strategies alone.

Cell viability was one of the major factors for enhanced PGL production. By combining both strategies, 80% of the cells were viable by the end of the fermentation, with an average of 91.33% in the second stage of fermentation, which was higher than keeping the residual methanol at a constant level of 12 or 16 ml/l. After the transition phase the cell viability had increased to 100% (Fig. 3d), which resulted in high PGL production of 1,634.78 U/ml between 80 and 90 h during the second stage of the induction phase. The PGL activity reported in the initial second stage was 492.75 U/ml compared to 16 ml/l methanol that resulted in 507 U/ml (Fig. 3e), but at 80 h PGL production increased a lot. It was concluded that the decrease in cell viability in the fed batch could be due to either carbon source limitations or buildup of toxic metabolic by-products [42], but still a small fraction of cell lyse. With our novel strategy,

Table 1 The highest values obtained for various parameters with control (pH 5.5 for the entire bioreaction), two-stage pH control and constant methanol feed at 12 and 16 ml/l, and by combining the two strategies

Parameters (maximum values achieved during the bioreaction)	Control	Two-stage pH control 5.5–4.5 at constant methanol feed (10 ml/h)	Online methanol feeding at pH 5.5		Novel strategy with both combinations
			12 (ml/l)	16 (ml/l)	
Cell viability (%) at 85 h	80	87	80	87	90
Biomass (g/l)	122.6	150	140	134	162
Residual CH_3OH (ml/l) at 85 h	25.99	17.962	N/A	N/A	N/A
PGL activity (U/ml)	956.5	1,362.3	1,144	1,252.2	1,631
Protease activity (U/ml) at 85 h	0.15	0.08	0.2	0.16	0.12
AOX activity (U/ml)	837.2	1,170	632.6	1,097.3	1,473.2
ATP, ADP and AMP (g/l)	0.9, 0.028, 0.2	1.3, 0.043, 0.4	0.9, 0.03, 0.22	1.2, 0.04, 0.28	1.6, 0.06, 0.6
Methanol consumed (l)	1.2 L	1.2 L	0.8 L	1.1 L	1.26 L

the viability remained high, which was an indication of lower cell metabolites and proper utilization of carbon sources. For the novel strategy, the ATP levels achieved were much higher than both the strategies, registering a maximum ATP concentration at 1.6 g/l, ADP at 0.06 g/l and AMP at 0.6 g/l at 90 h in the second stage of the induction phase (Fig. 3f). To keep the intracellular pH neutral, large amounts of ATP are consumed by the membrane-bound ATPase [37] as energy in the form of ATP is required for the secretion of recombinant proteins [45]. During the transition stage of glycerol feed the ATP, ADP and AMP reserves were enhanced. At a higher methanol concentration, much of the energy was used in the transportation of secreted proteins from the reticulum system to the Golgi complex [46]. This energy utilization, if not replenished during the induction phase, has been seen to cause translational errors and ultimately leads to truncated proteins [47] because when the cells are shifted from glycerol to methanol induction the morphology of cells changes [48].

This combined strategy utilized less methanol (Table 1), and the shift in the transition phase resulted in higher production. Although the amount of glycerol added was very small, it had a positive effect on *P. pastoris*. Such a strategy has never been reported before. This strategy also provided us the understanding that the time wasted as transition phase after the growth phase can be very helpful if strategically introduced during the induction phase. Our findings showed that to enhance the PGL or any other protein related to *P. pastoris*, it is important to divide the induction phase into two stages: a lower methanol concentration followed by an increased methanol concentration stage. This not only helps to increase the productivity, but can also lead to lower costs, higher AOX activity, lower protease activity and higher cell viability, which can be applicable for industrial-scale productivity.

Acknowledgments The funding for this research was supported by the National Outstanding Youth Foundation of China (no. 20625619), the Key Project of Chinese National Programs for Fundamental Research and Development (973 program) (no. 2007CB714306, no. 2007CB714303) and the Program for New Century Excellent Talents in University (NCET-07-0378).

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