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Effect of post-induction nutrient feed composition and the use of lactose as inducer during production of thermostable xylanase in *Escherichia coli* glucose limited fed-batch cultivations

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ABSTRACT

Escherichia coli is a microorganism routinely used for the production of heterologous proteins. Overexpression of a xylanase gene (Xyn10AΔNC), originating from the thermophile *Rhodothermus marinus*, cloned under the control of the strong T7/lac-promoter, in a defined medium (mAT) using a substrate limiting feed strategy, was however shown to impose a significant metabolic burden on the host cells. This resulted in a decrease in cell growth and ultimately also in decreased target protein production. The investigation hence centres on the effect of some selected nutrient feed additives [amino acid (Cys) or TCA-intermediates (citrate, succinate, malate)] as a strategy to relieve the metabolic burden imposed during the feeding and post-induction phases of these substrate (glucose) limited fed-batch cultivations. Use of either succinic acid or malic acid as feed-additives resulted in an increase in production of approximately 40% of the heterologous thermostable xylanase. Furthermore, use of lactose as an alternative inducer of the T7/lac-promoter, was also proven to be a suitable strategy that significantly prolonged the heterologous protein production phase as compared to when induced by isopropyl β-D-thiogalactopyranoside (IPTG).

[**Keywords:** thermostable, xylanase, lactose, pulsed feeding strategy]

INTRODUCTION

Heterologous protein production in *Escherichia coli* is widely used to obtain large amounts of proteins, which are not easily available from the wild-type organism. The production levels are affected by numerous process factors, such as cultivation mode, composition of the medium, time of induction (with respect to cell mass concentration) and duration of the production phase (1,2). Due to the lack of a natural secretion mechanism in *E. coli*, concentration of the produced protein is to a great extent proportional to the cell concentration, and processes designed to yield high cell densities are therefore beneficial. In addition other factors, such as host cell-vector interactions, plasmid stability and cellular stress responses (3-5) need to be considered. As a result, research to optimize the protein production is complex and encompasses the disciplines of both molecular biology and engineering, and numerous strategies are thus applied to maximize the production of recombinant proteins in *E. coli* (6-8).

Metabolic stress in *E. coli* is often proposed to be a reason for decreased yields of the target protein during its gene expression, which can result in the redirection of the cellular metabolism (9) and a decreased growth rate (6-10). This has in some cases been reported to occur due to the addition of the inducer IPTG (11,12) but is also more generally found during post induction phases especially when induction is initiated after a certain critical cell-mass concentration, possibly due to absence of necessary metabolites. It has for example previously been established that depletion of certain amino acids in the medium significantly influenced the expression of recombinant proteins (2,13). This lack of resources in the cell are likely due to the increased protein production rate, in combination with the fact that

the heterologous protein often has a different amino acid composition compared to that of average *E. coli* proteins. This can result in limitations in certain key amino acids that hence act as bottlenecks and control the protein production rate. Consequently, addition of the deficient amino acids to the growth medium has been reported to circumvent a decreased production of the recombinant protein in the late post-induction phase (2,13). Such additions can also circumvent reduced tRNA levels during translation, a factor that has previously been observed during amino acid limitation (14) and resulted in conditions with amplified proteolytic activity in the cell to replenish amino acid resources (15). Released amino acids from nonessential proteins may in this way be incorporated into essential proteins that are required for the cell survival (16).

This investigation hence centers on the effect of nutrient additives from the TCA-cycle (present during the fed-batch and post-induction phase) on recombinant protein production in *E. coli* grown using a substrate limited fed-batch strategy, designed to control the feed in order to keep glucose below the critical value for overflow metabolism (17). By analyzing protease activity, a measure on the induction related stress response is also collected. In addition, the effect of the choice of inducer (IPTG vs. lactose) on both the recombinant protein production and on the induction related stress response in cells grown by this fed-batch strategy is investigated. For these purposes high cell density fed-batch cultures of *E. coli* producing thermostable xylanase (18), originating from the thermophilic bacterium *Rhodothermus marinus* (19), were analyzed. The results are expected to give new complementary information regarding the protein-synthesizing machinery of the cell in order to explain the cell behavior as the recombinant protein-production proceeds.

MATERIALS & METHODS

Bacterial strain, plasmid and inoculum preparation

Escherichia coli strain BL21(DE3) was used as the host microorganism. The plasmid was derived from the vector pET25b(+) (Novagen, Madison, WI). The construction of plasmid encoding the xylanase (Xyn10A Δ NC) is described in (20). Xyn10A Δ NC consists of signal peptide (21aa) and the catalytic module (322aa) and has a short vector derived stretch of 15 amino acids in the C-terminus. Expression is in all cases under the control of the T7/lac promoter. Inoculum was prepared using 100 ml of defined mAT-medium [Table 1 (excluding IPTG & antifoam)]. The salts, glucose and trace elements were sterilized separately at 121 °C for 20 minutes and aseptically pooled into a 1 L baffled Erlenmeyer flask. Mid-log cultures (1 mL), in 20% glycerol (stored at -80 °C), containing the *E. coli* clone, was used to inoculate the flask which was subsequently incubated at 30 °C for 12 h at 125 rpm on a rotary, water bath shaker (Heto, Allerød, Denmark).

Experimental set-up and cultivation conditions

Fed-batch cultivations were performed using a 3 L fermentor (Chemoferm FLC-B-3, Hägersten, Sweden) with an initial medium volume of 2 L. The salts were sterilised in the vessel at 121 °C for 45 minutes, thereafter sterile MgSO₄, glucose and trace elements were aseptically added. Ampicillin (see Table 1) was added by sterile filtration. Data capturing of dissolved oxygen concentration (%DO), feed-pump control, stirrer speed (rpm) and off-gas were conducted with the SattLine control system (Alfa Laval Automation AB, Malmö, Sweden). Cultivation temperature was controlled at 37 °C and the pH was maintained at 7.0 by titration with 6.7 M aqueous ammonia. Dissolved oxygen concentration was measured using

a polarographic electrode calibrated to 100% at 1100 rpm at 37 °C and zeroed by sparging sterile nitrogen into the vessel. The DO was controlled at 30% saturation using a gain-scheduled PID controller connected to stirrer speed, as described in (17). A pulse feeding strategy (21) was employed using the varying nutrient feed solutions defined in Table 1. A Tandem dual gas system (Adaptive Biosystems, Luton, UK) was used for off-gas analysis. Induction was carried out by IPTG or lactose. Fermentations and assay conditions were standardised to avoid any bias and to allow accurate and reproducible comparison. Furthermore, selection of appropriate precursor supplementation concentrations was based on the highest concentrations of organic acids obtained during uninduced control cultivations.

Sampling and sample treatment

For intracellular enzyme activity, total protein estimation and SDS-PAGE, samples were centrifuged ($11,000 \times g$, 15 min, at room temperature) and the resulting cell pellets were dissolved to the original volume in 20 mM Tris-HCl pH 7.5 and disrupted by ultrasound (90 s, cycle 0.5) with a sound intensity of 230 W/cm^2 using UP400S sonicator (Dr. Hielscher GmbH, Stahnsdorf, Germany), equipped with a 3 mm titanium probe. After sonication the samples were centrifuged ($11,000 \times g$, 15 min, at room temperature) and the supernatants were stored on ice or kept frozen ($-20 \text{ }^{\circ}\text{C}$) until analysis.

For extracellular enzyme activity (i.e. analysis of enzyme activity in the fermentation broth as a result of lysis or cell disruption), samples were centrifuged ($11,000 \times g$, 15 min, at room temperature) and the resulting supernatants were stored on ice or kept frozen ($-20 \text{ }^{\circ}\text{C}$) until analysis.

For HPLC analysis of organic acids and glucose, samples were centrifuged ($11\,000 \times g$, 5 min, at room temperature) and the resulting supernatants were filtered (0.2 μm) into vials and stored ($-20\text{ }^{\circ}\text{C}$) until analysis.

Analytical Methods

Optical density and Cell dry weight determination

The optical density was determined at 620 nm. Samples exceeding an OD value of 0.5, were appropriately diluted with 0.9% (w/v) NaCl before determination.

Triplicate cell dry weight (CDW) determinations (4 ml sample each) were made after drying overnight at $105\text{ }^{\circ}\text{C}$ as described in (13).

Enzyme activity

Xylanase activity was determined using the DNS (3,5-dinitrosalicylic acid) method (22) under the conditions described in (13). Xylanase activity was expressed in units (U) which is defined as the amount xylanase required to liberate 1 μmol of reducing sugar equivalents per min under standard conditions.

Estimation of total protein

Protein concentration was determined using the bicinchoninic acid (BCA) method (Sigma) with bovine serum albumin (0.2-1.0 mg/ml) as a standard.

Organic acids

The concentration of organic acids was determined by HPLC using an Aminex HPX-87H column (Bio-Rad, CA, USA). The column temperature was set at $30\text{ }^{\circ}\text{C}$ at a wavelength of 210 nm and a mobile phase (0.005 M H_2SO_4) using a flow rate of 0.5 ml/min. An organic acid standard (Bio-

Rad: Catalog no. 125-0586) which enabled the quantification of citric acid, succinic acid, and malic acid was used for calibration.

Glucose estimation

The residual glucose concentration was determined using a Waters Breeze HPLC System equipped Breeze Software and a Water 2410 refractive index detector (Waters Corporation, Milford, MA). The internal temperature of the RI-detector was 45°C and the external temperature was 60°C using 0.005 M H₂SO₄ as elute. Glucose standards (0.1-10 g/L) were used for calibration.

TABLE 1: Medium composition for batch and nutrient feed-solutions

| Ingredient | Concentration | |
|--|---------------------------------------|--------------------|
| (NH ₄) ₂ SO ₄ | 2 | g L ⁻¹ |
| K ₂ HPO ₄ | 14.6 | g L ⁻¹ |
| NaH ₂ PO ₄ 2H ₂ O | 3.6 | g L ⁻¹ |
| (NH ₄) ₂ H-citrate | 0.5 | g L ⁻¹ |
| Glucose | 10 | g L ⁻¹ |
| 1M MgSO ₄ | 2 | ml L ⁻¹ |
| *Trace elements | 2 | ml L ⁻¹ |
| Ampicillin | 0.1 | g L ⁻¹ |
| Antifoam (Adekanol) | 0.1 | ml L ⁻¹ |
| Inducer | 1 mM (IPTG) or 13 mmol/g CDW(Lactose) | |
| Feed (F) | | |
| Glucose | 500 | g L ⁻¹ |
| 1M MgSO ₄ | 50 | ml L ⁻¹ |
| Trace elements | 10 | ml L ⁻¹ |
| Feed (F_{cit}) | | |
| Feed F + Citric acid | 2 | g L ⁻¹ |
| Feed (F_{suc}) | | |
| Feed F + Succinate | 2 | g L ⁻¹ |
| Feed (F_{mal}) | | |
| Feed F + Malate | 2 | g L ⁻¹ |
| Feed (F_{cys}) | | |
| Feed F + Cystein | 2 | g L ⁻¹ |

SDS-PAGE

Protein production was analysed by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to (23) and stained with Coomassie Brilliant Blue G250 (Merck, Darmstadt, Germany). Densitometric measurements to estimate the percentage of recombinant protein were performed using a Gel Doc 2000 system (Bio-Rad). The sum of the light intensity from individual bands in the lane (last hour of induction) was divided by the light intensity of the band corresponding to recombinant xylanase.

Qualitative detection of protease activity by CPAGE

Detection of protease by *CPAGE* (14) was performed using the conditions described previously (13).

RESULTS

Previous results (13), using the same host-vector system, the same fed-batch strategy, and a feed consisting solely of glucose, magnesium and trace elements (F in Table 1) were indicative of a nutrient limitation during the post induction phase as xylanase activity significantly decreased 2 h after induction. This decrease was avoided when the amino acid Glu (over-represented in the xylanase compared to average *E. coli* proteins) was added to the nutrient feed and it was concluded that this addition alleviated limitation of building blocks needed for the production of the recombinant protein. In this work, the cause of the post-induction nutrient limitation (monitored by following xylanase production, detectable protease activity, or the level of TCA-cycle intermediates) is further investigated using feed supplementation with: I) an amino acid (Cys) not over represented in the target protein, to analyse if addition of any amino acid affects target protein production and II) selected TCA-cycle intermediates (Table 1), which are

organic acids and precursor molecules with a potential to affect recombinant protein production. In addition the effect of the inducer-choice (IPTG vs. lactose) at glucose limited conditions was evaluated.

TABLE 2: Protease detection at two stages of the fed-batch cultivation, in the pre-induction feeding phase, and in the post-induction phase 2 h after induction. Detected protease activity is indicated (+) (-) = not detected

| Type of feed (Inducer) | Protease detection by CPAGE | |
|---------------------------|-----------------------------|----------------------|
| | Pre-induction phase | Post-induction phase |
| F (IPTG) | - | + |
| F (uninduced) | - | - |
| F (Lactose) | - | - |
| Fcit (IPTG) | - | + |
| Fsuc (IPTG) | - | - |
| Fmal (IPTG) | - | - |
| Fcys (IPTG) | - | - |

Feed strategy and feed-supplementation

The substrate limiting feeding strategy (17) was applied in all cultivations. The start of the feeding phase was commenced upon depletion of the initial glucose, detected as a peak in the DO signal, and the feed rate was gradually increased by the control algorithm until maximum stirrer speed was obtained. Thereafter, the feed rate was mainly limited by cell metabolism as seen in the oxygen response to feed-pulses, and in the later phase a safety net gradually decreased the feed rate to maintain aerobic conditions, keeping the stirrer speed at its maximum (21). Totally seven fed-batch cultivations, all controlled by the feeding strategy described above were run. The batch phase conditions were similar in all cultivations, and the cell dry weight obtained at the transition from batch to fed-batch

phase did not significantly vary between the cultures (6.5 ± 0.7 g/L). At this stage, the substrate-limiting feed-control strategy was initiated, and the variation in feed-composition introduced. This consisted of feed-supplementation (2 g L^{-1}) with one of the following components: the amino acid cysteine or one of the organic acids: citric acid, succinic acid, malic acid, in each of four cultivations (where stated). The remaining three cultivations were fed with the originally defined feed (F in table 1), and variation was instead introduced at the point of induction (one induced by IPTG, one by lactose, and one uninduced).

Six of the seven cultures were hence induced 2.5 h after the feed-start: five (four with feed-supplementation's and one with the originally defined feed, F) by a single addition of the inducer IPTG (1 mM), and one (fed with the originally defined feed, F) with the alternative inducer lactose (13 mmol/g CDW). The seventh cultivation was an uninduced control cultivation (also fed with the originally defined feed F in table 1), run to establish a baseline comparison for the induced cultivations mentioned above. Induction was initiated at approximately the same cell mass concentration (21.4 ± 1.2 g/L), in all six cultivations. The protein production phase in the cultivations induced by IPTG was limited to a time-period of 3 h, based on previous results (6,13), while the lactose induced production proceeded for 6 h. Lactose induction was analysed as a low cost alternative, suitable with this type of substrate-limited feed control strategy, as the concentrations of glucose are proven to be low throughout the fed stages of the cultivation (6,13,17).

Analysis of organic acids, and biomass production

HPLC analysis allowed the quantification of, citric acid, succinic acid, and malic acid in the fermentation samples [from unsupplemented

cultivations and from those supplemented with the above organic acids (Table 1)]. The results from the sample analysis were subsequently evaluated in order to ascertain the effect of the limited substrate feeding on their concentration levels during cultivation (Fig. 1A-C). A comparison between the uninduced cultivation and cultivations induced with IPTG (both fed with the originally defined feed) showed an almost complete exhaustion of the concentration of both citric acid (Fig. 1A) and malic acid (Fig. 1B) during the post induction phase. Succinic acid was not exhausted, but concentrations (Fig. 1C) were reduced to approximately 60% during the post induction phase compared to the uninduced culture. In the lactose-induced cultivation, the complete exhaustion was avoided, which could be a consequence of the lactose addition, and hence higher level of available carbon-source. As expected (Fig. 1B & C), the residual concentrations of succinic acid and malic acid were higher during the pre-induction (fed-batch starts at 5.5 h) and post induction phases when nutrient feeds F_{suc} and F_{mal} are employed, respectively, as opposed to use of an unsupplemented feed (F). Although the citric acid concentration decreased during the post-induction phase also when using feed-supplementation (F_{cit}) it only amounted to 30% compared to the 100% reduction using feed F. Thus, the previously observed depletion during the post-induction phase was circumvented. Accumulation of the added organic acid towards the end of the post-induction phase, was only observed using succinic acid supplementation, indicating direct use in metabolism, providing an increased flux through the TCA cycle or use as precursors for transamination reactions. It was also observed that there was an increase in cell mass production rate when either F_{cit} or F_{cys} (Fig. 2B) was used as feed as compared to using feed F_{mal} or F_{suc} (Fig. 2A). This was an indication that citrate and the amino acid cysteine were metabolites used

directly for host specific biosynthesis (cellular components) due to a final cell dry weight concentration of approximately 12.3% higher being observed when feeds F_{cit} and F_{cys} were employed (Fig. 2 B). In line with previous results using the substrate limited control strategy (13,17), offline analysis indicated no glucose accumulation with concentrations maintained below 0.2 g L^{-1} during the feed-phase even when employing metabolite supplemented feeds.

The effect of nutrient feed supplementation's on xylanase activity and on detectable protease activity

The presence of either succinic acid or malic acid increased xylanase production levels by approximately 41.6% [compared to the highest xylanase activity attained using unsupplemented feed, F (Fig. 3)]. Moreover, the previously observed decrease in xylanase activity (using unsupplemented feed, F) was alleviated when these precursors were present in the nutrient feed. In contrast, the presence of citric acid or the amino acid cysteine in the feed did not show any effect on enzyme production levels and displayed an enzyme activity curve similar to cultivations employing the unsupplemented feed (Fig. 3). Xylanase activity measurements of the fermentation broth revealed an average of 9.1 % of the total measured intracellular activity irrespective of the nutrient feed used. A previous investigation (20) showed that the xylanase activity was located both in the periplasm and intracellular, but that the major part of the activity was intracellular despite the presence of the signal peptide. Our current results also show that a similar degree of either cell lysis or leakage of the enzyme occurs during the cultivation and is independent of the nutrient feed composition. Furthermore, protease was detected in the citric acid supplemented fed culture (F_{cit}) at a similar point (2 h) as in the unsupplemented culture (F) during the post induction phase (Table 2). This

further demonstrates that the presence of citric acid or cysteine was not effective for recombinant xylanase production in this study, but that it may be metabolised for host specific biosynthetic reactions.

The effect of the alternative inducer lactose on xylanase activity and on detectable protease activity in substrate limited fed-batch cultivations

The applicability of lactose for gene expression control was assessed in this type of glucose limited fed-batch cultivations employing unsupplemented feed (F). In order for the induction efficiency of lactose to be equivalent to that of IPTG, both the lactose uptake and the conversion to the true inducer allolactose, needs to be efficient. The first requirement was considered fulfilled by the low levels of glucose, kept as a consequence of the control strategy, which should allow uptake. Therefore, a one-point induction strategy involving the introduction of lactose to the fermentor was performed. This resulted in a metabolic shift (Fig. 4) decreasing the glucose nutrient feed rate during the post-induction phase in order to maintain 30% dissolved oxygen saturation, and was indicative of an increased lactose uptake rate. It was shown that, although the recombinant protein production start is delayed approximately 30 min as compared to the IPTG-induced culture, the rate of product formation appears to be approximately the same once the production proceeds. This is in accordance with previous data collected from lactose induced fed-batch cultures fed using an exponential scheme but assuming a low specific growth rate (6), and may be a reflection of initial conversion time from lactose to allolactose. Moreover, the specific xylanase activity (Fig. 5A) continually increased during the entire post-induction phase (6 h). Judging from SDS-PAGE (Fig. 5B), the recombinant xylanase constituted approximately 30 % of the total protein at the end of the cultivation. Also,

protease activity was undetected during the pre- and post-induction phases (Table 2). Finally, the pronounced reductions in citric acid, malic acid and succinic acid (Fig. 1A-C) were avoided during the post induction phase. Taken together, this indicates a significant reduction in the metabolic burden imposed during recombinant protein expression when lactose is employed as an inducer under otherwise substrate limited conditions.

DISCUSSION

In this work we have explored the effect of selected organic acids from the TCA-cycle on the production of a recombinant protein in *E. coli*, grown using a substrate (glucose) limited fed-batch strategy. In addition supplementation with an amino acid (not over-represented in the recombinant protein compared to average *E. coli* proteins) was investigated. Expression of recombinant proteins has previously been shown to be strongly affected by amino acid deficiencies (2,8,13). The amino acid deficiencies has in turn been suggested to lead to reduced tRNA levels during translation, creating metabolic conditions that ultimately result in induction-related stress responses, such as increased protease activity, which (24) towards already synthesized recombinant proteins (in order to supply amino acids for the host cell metabolism) ultimately decreases productivity. Over-expression of recombinant proteins will hence cause a diminished flow in the TCA cycle through the withdrawal of intermediates that serve as precursors for further biosynthesis (25). Ten amino acids (4 of the Glu-family, and 6 of the Asp-family) are biochemically derived from TCA cycle intermediates (Fig. 6), which imposes an additional metabolic burden during recombinant protein expression that may result in a decreased growth of the host cell or poor expression of the desired protein due to the increase in protein synthesis rate redirecting the carbon flux

from anabolic to energy-generating catabolic pathways (26). Our results showed that the metabolic load caused by expression of heterologous xylanase in minimal medium exceeded the host's capacity thereby resulting in a loss of final enzyme yield, which was likely caused by amino acid misincorporation and protease degradation (eventually in combination). Another indication of metabolic load was the decrease in key metabolic intermediates during the post-induction phase accompanying the decrease in xylanase activity. Therefore, in order to better synchronize the metabolic need the feed strategy was modified to include both control of the nutrient feed rate, and supplementation of the feed with amino acids/other metabolites in order to reduce biosynthetic precursor demand. This was the reason for selecting 3 intermediates (citrate, succinate and malate) from the TCA-cycle as feed supplements in order to establish a balance between heterologous and host specific protein biosynthesis in order to enable the production phase to be extended thus concomitantly increasing the amount of heterologous protein produced. It has previously been shown that supplementation with the amino acid Glu, (which is over-represented in the xylanase produced in this work) resulted in improved production yield during the late production-phase (>2 h production) (13). It was however unclear if the production improvement was due to a balancing (based on the amino acid residue composition of the xylanase), or if it was a relieving effect caused by the presence of any precursor metabolite. During this investigation it was found that not all post-induction nutrient supplementation's directly ensured an increased expression of the gene encoding the heterologous protein. This could be seen with regards to addition of the amino acid cystein, an amino acid with a metabolic precursor (3-phosphoglycerate) in the Embden-Meyerhof pathway, but which opposed to glutamate is not over-represented in the heterologous

xylanase. Use of this supplementation, although it did not improve recombinant protein production, instead led to an increased biomass production. A similar effect was also seen using the TCA-intermediate citrate. So despite lacking an effect in recombinant protein production, these additions appeared to have been utilized for host specific biosynthesis. On the other hand the presence the TCA cycle intermediates malate or succinate, respectively, resulted in a pronounced increase of the heterologous xylanase during the late (> 2 h) production phase. Thus it can be seen that during the post induction phase low levels of TCA cycle intermediates have a direct influence on heterologous protein production levels and host specific biosynthesis. However, these limitations may be overcome by using an optimized nutrient feed during the post-induction phase.

Metabolic balancing during the production phase to circumvent loss of produced protein can be achieved in different ways. The nutrient supplementation way described above is affecting the translational level, as it involves manipulation of the amount available nutrients in the feed in order to achieve metabolic balancing. Alternative strategies can however also be used, such as modulation of the gene dosage (on replication level) by use of vectors with varying copy numbers, or modulation of the transcription either by use of promoters of different strength, or by inducer tuning of a strong promoter (27, 28). The latter offers an easier alternative from a process-engineering point of view, as tuning of the expression rate is also possible for example by use of lactose as an inducer. The T7/*lac* promotor using IPTG to induce protein expression is one of the most powerful and commonly used systems employed for heterologous protein production due to its specificity and control (29). Although it's a strong promotor, use of IPTG is however not cost effective for industrial scale

and the inducer has in some cases been reported to be toxic to the cells (30). The use of lactose may hence provide an inexpensive alternative for the induction of *lac*- (and related) promoters, and it can simultaneously serve as an inducer and as a carbon source (6, 31-34).

In conclusion, the findings of this study suggest that there is a strong dependence of nutrient feed composition in the post induction phase for efficient protein synthesis. Furthermore, lactose is proven to be a suitable inducer for expression under the control of the *lac* promotor using the substrate limited fed-batch strategy, indicating the potential for production of thermostable xylanase from *R. marinus* by an *E. coli* expression system.

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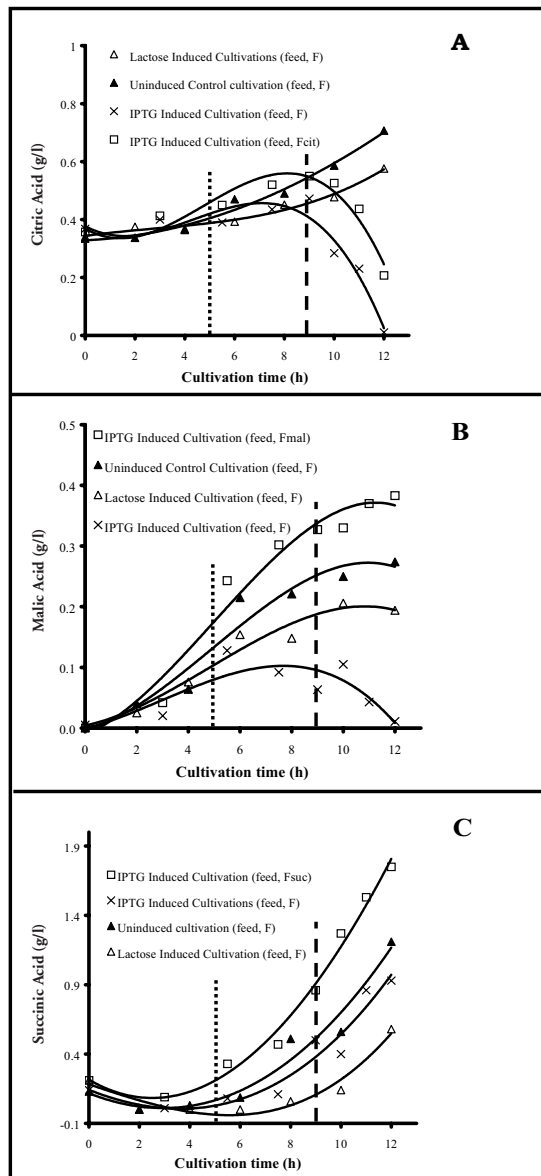


FIG. 1A-C. HPLC analysis of fermentation samples for the quantification of organic acids. A) measured citric acid concentration. B) measured malic acid concentration. C) measured succinic acid concentration. Nutrient feed started at 5 h (dotted line) and induction at 9 h (dashed line).

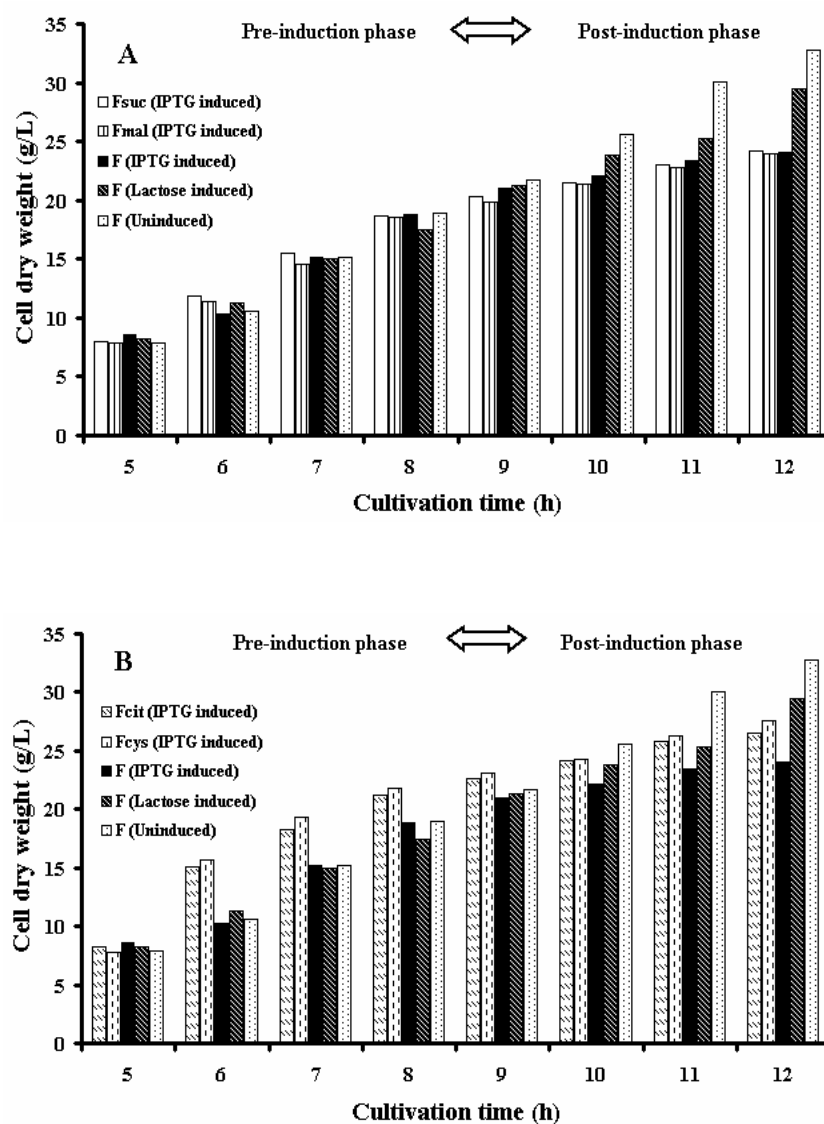


FIG. 2. Cell dry weights attained in fed-batch cultivations shown from the start of the feeding phase employing varying nutrient fed composition. A) demonstrates the effect of malic and succinic acid on cell mass production. B) demonstrates the effect of citric acid and the amino acid cystien on cell mass production. For comparative purposes only the first 3 hours using lactose as an inducer is shown. Expression of the xylanase gene is induced at cultivation time 9 h.

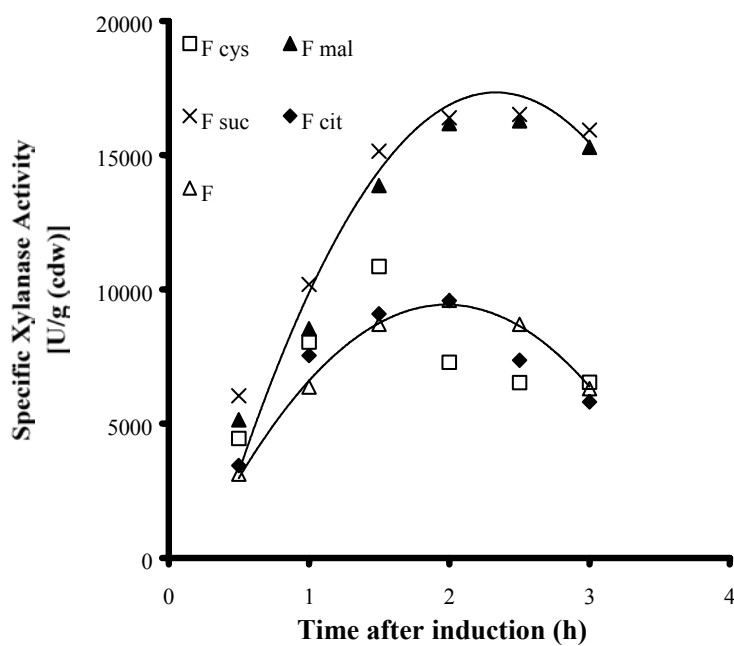


FIG. 3. The effect of varying nutrient feed composition on heterologous thermostable xylanase production during the 3 h production phase in the substrate (glucose) limited high cell density fed-batch cultivations using IPTG as an inducer. Trend-lines were fitted using data points of F suc and ΔF.

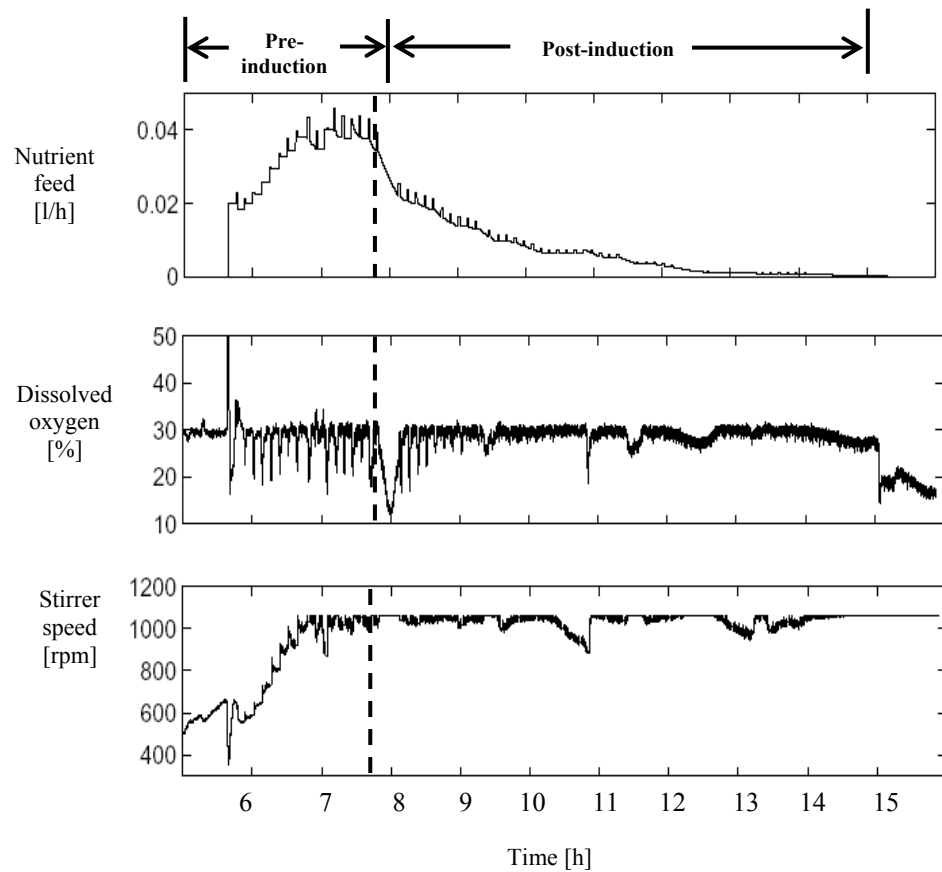


FIG. 4. High cell density fed-batch cultivation (HCDC) of recombinant *Escherichia coli* strain BL21(DE3) encoding thermostable xylanase (Xyn10A Δ NC). Nutrient feed started at 5 h after inoculation and lactose was used as an inducer (dashed line) at 7.8 h.

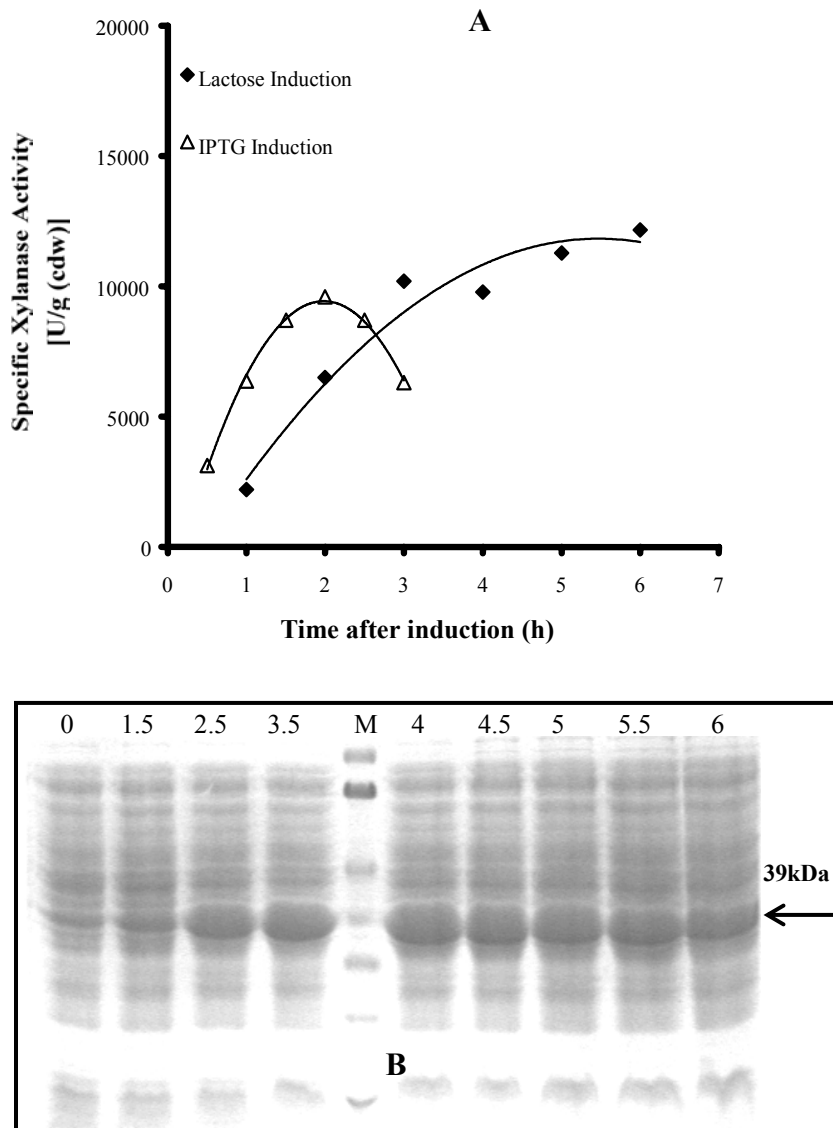


FIG. 5. Specific xylanase activities expressed in units (U) per gram cell dry weight. A) comparison of xylanase activity using IPTG or lactose as an inducer. B) SDS-PAGE from post induction phase of the cultivation using lactose as an inducer. The arrow indicates the 39 kDa (Xyn10AΔNC) xylanase produced at various times after induction (0-6 hours). The position of the xylanase active band was confirmed by activity staining using a substrate containing overlay gel. (M) Corresponds to the molecular mass marker (113, 92, 53, 35, 29 and 22kDa).

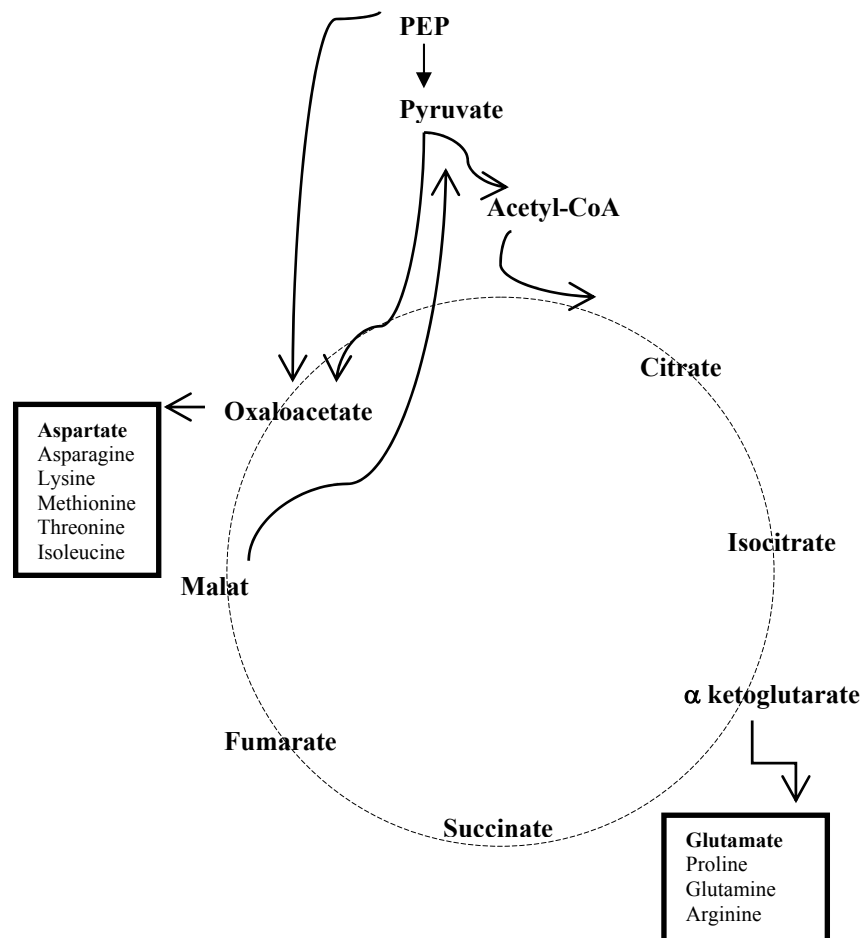


FIG. 6. Schematic representation of TCA cycle showing precursors used directly in the synthesis of amino acids.