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From laboratory to pilot plant *E. coli* fed-batch cultures: optimizing the cellular environment for protein maximization

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Abstract For recombinant protein production in *E. coli* fed-batch cultures, post-induction conditions have great influence in the quantity and quality of the product. The present paper covers the effect of different factors affecting the cellular environment in recombinant aldolase (rhamnulose-1-phosphate aldolase, RhuA) production. An operational mode employing an exponential addition profile for constant specific growth rate has been analyzed, in order to understand and define possible modifications with influence on post-induction cellular behavior. A constant addition profile has been demonstrated to render higher specific aldolase production than the exponential addition profile, probably due to a more constant environment for the cells. On the other hand, amino acid (leucine) supplementation has proven to increase protein quality in terms of activity units (U) per unit mass of RhuA (U mg^{-1} RhuA), alleviating metabolic overload. Based on the above, a production process was set up and scaled up to pilot plant. Resulting production was double that of a standard laboratory operation, 45,000 U L^{-1}, and almost all the protein retained the 6xHis-tag with the highest quality, 11.3 U mg^{-1} RhuA.

Keywords *E. coli* · Scale-up · Pilot plant · Aldolase · Post-induction feeding · Amino acid supplementation

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Introduction

Microbial protein production is one of the battle horses of modern Biotechnology. For relatively simple proteins with lack of post-translational modifications, *E. coli* is one of the preferred expression systems, especially for the production of industrially relevant enzymes [10, 23].

From a productivity point of view, high-cell density cultures can be obtained by fed-batch operation. Exponential feeding has been commonly employed to obtain a desired specific growth rate (μ) before induction of expression [10, 28]. In that case, the process is operated under glucose (or other essential substrate) limiting conditions, which has the advantage of continuously providing the required amount of nutrients to sustain growth and reach high productivities [19, 28]. Nevertheless, during induction of the target protein, overexpression under carbon source limiting conditions (like exponential feeding) can lead to cellular stress situations conducive to proteolysis, with the objective to obtain additional resources for growth and maintenance [7, 8, 11, 16], supporting both overexpression of recombinant protein and heterologous DNA replication. On the other hand, the high amino acid demand during protein synthesis imposes a metabolic overload to the microbial cell [4, 20]. This is especially important if the target protein has an amino acid composition profile significantly different that the mean of the *E. coli* cytoplasmic proteins, conducive to disturbances in the amino acid synthesis network [9, 15]. On the other hand, the shortage of precursors during induction of overexpression, as well as the abnormal accumulation of proteins, lead to stringent response [2, 6, 22, 26].

Thus, from a process development point of view, it is essential to define proper operation conditions that could alleviate the above-mentioned effects, by ensuring an

appropriate environment for cell growth and expression. Different publications have dealt with the definition of operational conditions to be scaled up, and minimization of the negative effect of foreign protein overexpression on growth and overall yields [10, 21, 23]. The suggested alternatives involve, among others, medium optimization by selective amino acid supplementation before induction [9, 13], and use of different feeding policies after pulse induction, such as linear or constant feeding [3, 27].

Our research group has been working on the production of recombinant aldolases using an auxotrophic strain during the last years, covering aspects such as production strategies [17], modeling and control [18]. As a consequence, a single isopropyl β-D-1-thiogalactopyranoside (IPTG) pulse induced fed-batch culture methodology was established. Starting from the knowledge of the system, the main objective of the present work was to develop an operational methodology for improving production that could be translated to pilot plant and industrial scale. The growth and induction strategies will be postulated, taking into account ease of implementation, moderate consumption of resources and ability to keep a favorable cellular environment. Improvements tested at bench scale will later be scaled to a pilot (50 L) production process.

Materials and methods

Bacterial strain and plasmids

A K-12 derived strain *E. coli* M15ΔglyA [pREP4] harboring the vector pQEαβrham was used for rhamnulose 1-phosphate aldolase (RhuA) overexpression. The system is based on glycine auxotrophy, avoiding the need of antibiotic supplementation to ensure plasmid stability [25]. Transcription of the *rhaD* gene is under the control of the strong T5 promoter in a low copy number plasmid derived from pQE-40 (Qiagen, Hilden, Germany), and aldolase is expressed as a fusion protein to a 6xHis tag, allowing further easy affinity purification.

Reagents

A stock solution of kanamycin (Sigma, St. Louis, Mo, USA) 100 mg mL^{-1} was prepared in milliQ water, filter sterilized and stored at 4 °C. Stock solutions of ampicillin (Sigma) 100 mg mL^{-1} were prepared in 50 % (v/v) ethanol, filter-sterilized, and stored at −20 °C. IPTG was purchased from Sigma and a 100 mM stock solution was prepared using milliQ water as solvent, filter-sterilized and stored at −20 °C.

Media composition

LB medium, with a composition of 10 g L^{-1} peptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl, was used for the preinoculum preparation.

A defined mineral medium (DM), utilizing glucose as the sole carbon source, was used for inocula, for shake flask experiments and for all bioreactor cultivation experiments.

The medium for shake flask cultures was composed of 5 g L^{-1} glucose, 2.97 g L^{-1} K_2HPO_4, 0.59 g L^{-1} KH_2PO_4, 0.46 g L^{-1} NaCl, 0.75 g L^{-1} $(NH_4)_2SO_4$, 0.11 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.006 g L^{-1} $FeCl_3$, 0.025 g L^{-1} thiamine, 0.001 g L^{-1} $CaCl_2{\cdot}2H_2O$ and 0.8 mL L^{-1} of trace elements solution.

The batch phase of bioreactor cultivations was composed of 20 g L^{-1} glucose, 11.9 g L^{-1} K_2HPO_4, 2.4 g L^{-1} KH_2PO_4, 1.8 g L^{-1} NaCl, 3 g L^{-1} $(NH_4)_2SO_4$, 0.45 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.02 g L^{-1} $FeCl_3$, 0.1 g L^{-1} thiamine, 0.004 g L^{-1} $CaCl_2{\cdot}2H_2O$ and 2.86 mL L^{-1} of trace elements solution.

The feed medium for bench scale high-cell-density fermentations consisted of 487 g L^{-1} glucose, 9.56 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.5 g L^{-1} $FeCl_3$, 0.34 g L^{-1} thiamine, 0.089 g L^{-1} $CaCl_2{\cdot}2H_2O$, 63 mL L^{-1} trace element solution and 0.5 mL L^{-1} of antifoam (Sigma).

The feed medium for pilot plant culture contained: 300 g L^{-1} glucose, 6.75 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.3 g L^{-1} $FeCl_3$, 0.3 g L^{-1} thiamine, 0.06 g L^{-1} $CaCl_2{\cdot}2H_2O$, 40 mL L^{-1} trace element solution and 0.5 mL L^{-1} of antifoam (Sigma).

The trace element solution composition contained (g L^{-1}): 0.042 $AlCl_3{\cdot}6H_2O$, 1.74 $ZnSO_4{\cdot}7H_2O$, 0.16 $CoCl_2{\cdot}6H_2O$, 1.6 $CuSO_4$, 0.01 H_3BO_3, 1.42 $MnCl_2{\cdot}4H_2O$, 0.01 $NiCl_2{\cdot}6H_2O$, 0.02 Na_2MoO_4 [18].

Phosphates were not included in the feeding solution in order to avoid co-precipitation with magnesium salts. Instead, a concentrated phosphate solution containing 500 g L^{-1} K_2HPO_4 and 100 g L^{-1} KH_2PO_4 was pulsed during the fed-batch phase when necessary to avoid their depletion.

Cultivation conditions

Inocula preparation

Pre-inocula were grown from glycerol stocks in 10 mL LB medium with ampicillin 100 μg mL^{-1} and kanamycin 100 μg mL^{-1} for 14–16 h at 37 °C, 150 rpm. Inocula were grown in 2 × 100 mL shake flask cultures at 37 °C, 150 rpm in defined medium (DM) until OD_{600nm} was 1.2 (4.5 h).

Standard fed-batch cultures

For standard bioreactor fed-batch experiments, 80 mL of inoculum culture were transferred to the fermentor containing 720 mL of defined medium. Cultures were carried out using a Biostat® B bioreactor (Sartorius, Goettingen, Germany) equipped with a 2 L fermentation vessel. The pH was maintained at 7.00 ± 0.05 by adding 15 % NH_4OH solution to the reactor. The temperature was kept at 37 °C. The pO2 value was maintained at 50 % of air saturation by adapting the stirrer speed between 350 and 1,120 rpm and supplying air (enriched with pure oxygen when necessary) at a space velocity of 2 vvm. The end of the batch phase was identified by a reduction in the oxygen consumption rate and an increase in pH.

Once the substrate was consumed, a fed-batch phase started supplying the feedstock solution with a microburette MICRO BU 2030 (Crison Instruments, Alella, Catalunya, Spain) equipped with a 2.5 mL syringe (Hamilton, Reno, NV, USA). The specific growth rate (μ) was kept constant using discrete feed additions as an approximation to a predefined exponential feeding profile [5], according to:

$$\Delta V = \frac{X_0 \cdot V_0}{S_f \cdot Y_{X/S\,ap}} \cdot (\exp(\mu_{set} \cdot t) - 1)$$

where: ΔV, volume to be added at time t; X_0 and V_0, biomass concentration and liquid volume in the fermentor at time of the previous addition; S_f, glucose concentration in the feed solution; $Y_{X/S\ ap}$, apparent yield biomass/glucose; μ_{set}, desired specific growth rate; t, time since previous addition. The value of μ_{set} used was 0.22 h^{-1}.

Induction of the cultures was carried out with an IPTG pulse. The exponential feeding profile was maintained until glucose started to accumulate in the bioreactor. To avoid glucose concentrations higher than 0.5 g L^{-1}, medium feeding was manually and periodically interrupted and restarted as a function of glucose off-line measurements.

Modifications of the standard procedure

– Model based culture. A modification of the standard operation was to use predictions of a mathematical model to anticipate glucose accumulation acting on the feeding flow rate. The procedure was like the standard, but feeding was stopped and restarted according to model predictions instead of from off-line analysis. This strategy allows a closer approximation to exponential feeding than the standard one, due to more frequent interruption sequences, improving process automation and reducing nutritional requirements (35 % less nutrients) [18].
– Constant feeding flow rate. Another modification of the post-induction standard operation was to use a constant feeding flow rate during the induction stage.
– Amino acid supplemented cultures. Standard cultures with amino acid supplementation were performed by adding the required amounts of each of the following amino acids: histidine (22 mg L^{-1}), tryptophan (17 mg L^{-1}), threonine (50 mg L^{-1}), leucine (70 mg L^{-1}) and glycine (60 mg L^{-1}).

Fed-batch fermentation at pilot scale

For pilot plant fermentations, a Biostat® UD50 bioreactor (Sartorius) with a total capacity of 50 L was employed. 200 mL of a 1.5 L laboratory scale culture were inoculated into 30 L of defined medium. Media composition, as well as batch and fed-batch operation mode, was similar to laboratory cultures. The pilot plant-specific conditions were as follows: (1) a programmable pump was employed for fed-batch feeding; (2) after reaching an OD of 60, a pulse of 2.7 g of leucine was added (representing 20 % of the necessary for the expected aldolase titer), and the culture was induced by an IPTG pulse of 476 mg (3 μmol IPTG g^{-1} DCW); (3) an approximately constant feeding profile was implemented after induction, corresponding to 0.5–0.4 g glucose g^{-1} DCW h^{-1}.

Downstream processing

The fermentation broth of laboratory scale cultures was centrifuged at 10,000 rpm for 20 min at 4 °C using a Beckman J2-21 M/E centrifuge. Harvested cells were resuspended in lysis buffer: 43 mM Na_2HPO_4, 7 mM NaH_2PO_4, 20 mM Imidazol, 300 mM NaCl (pH = 8) at a ratio of 1 mL buffer :0.3 g harvested cells. Resuspended cells were lysed by one-shot high-pressure disruption (Constant Systems LTD One Shot) at 2.57 kbar and at constant temperature of 4 °C. The crude cell lysate was centrifuged at 14,000 rpm for 35 min at 4 °C and cell debris was rejected. Enzymatic activity of the supernatant was measured, and sodium azide was added to keep a concentration of 0.02 % (w/w) to avoid biological degradation of the clear lysate.

For pilot plant production, culture broth was centrifuged at 10,000 rpm in a CSA-1 Gea Westfalia Separator. Harvested cells were resuspended in lysis buffer to reach an optical density (OD) of 100, and lysed in a continuous high pressure cell disruption system (Constant Systems TS5) at 2.57 kbar and a constant temperature of 4 °C.

RhuA purification-immobilization

Aliquots (10 mL) of clear lysate at the appropriate activity concentration were employed for one-step purification immobilization on Co-IDA support (Chelating Sepharose FF Amersham Biosciencies-GE Healthcare with Co^{2+} chelated onto it). One mL sample was used as reference and was kept under mild horizontal agitation at 4 °C. Its activity was measured both at the beginning and end of the immobilization process. The second sample (9 mL) was added to 1 mL of Co-IDA support, and the residual activity of the suspension and supernatant was measured until the adsorption–desorption equilibrium was reached.

Analytical procedures

Monitoring bacterial growth

Growth was followed by optical density measurements at 600 nm (OD_{600nm}). The samples were diluted in deionised water until the measurement was within the linear range of the spectrophotometer. The dry cell weight (DCW) was measured by centrifugation of aliquots of the broth. The pellets were washed twice with deionised water and dried at 110 °C until constant weight. As a result of a calibration curve, 1 OD_{600nm} was found to be equivalent to 0.3 g L^{-1} DCW.

Total cell number was determined using flow cytometry. All measurements were performed with a Guava EasyCyte Mini cytometer. Culture broth samples were diluted to a cellular concentration between 10^4 and 10^5 cell mL^{-1} (OD 10^{-5}–10^{-4}) to ensure proper counting. Samples were processed by quadruplicate and results expressed as the arithmetic mean.

For other determinations, one milliliter of culture was centrifuged. The supernatant was then used for glucose, organic acids, and ammonium and phosphate measurements. Glucose and organic acids were analyzed by HPLC (Hewlett Packard 1050) on an Aminex HPX-87H (Bio-Rad, Berkeley, CA, USA) column at 25 °C with IR detector (HP 1047) using 15 mM H_2SO_4 (pH = 3.0) as eluent at a flow rate of 0.6 mL min^{-1}. Ammonium and phosphates were determined by colorimetric kit assays (Dr. Lange, Basingstoke, UK) following the supplier instructions.

To quantify the product concentration during cultures, broth samples were withdrawn and centrifuged. The pellet was resuspended in 100 mM Tris·HCl (pH = 7.5) to a final OD_{600nm} = 3 for enzyme determination. Cell suspensions were placed in ice and sonicated using a Vibracell® model VC50 (Sonics & Materials, Newtown, CT, USA), with four 15 s pulses and 2 min intervals in ice between each pulse,. Cellular debris was removed by centrifugation and the clear supernatant was collected for product analysis.

Product quantification

Total protein content was determined using a Coomassie® Protein Assay Reagent Kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). To determine the percentage of RhuA amongst the rest of intracellular soluble proteins, 12 % polyacrylamide SDS-PAGE gels were performed in a Miniprotean® II instrument (Bio-Rad) according to the manufacturer's instructions and quantified by Kodak Digital Science® densitometry software.

Determination of RhuA activity was carried out as described previously [24]. One unit of RhuA activity was defined as the amount of enzyme required to convert 1 μmol of rhamnulose 1-phosphate in DHAP per minute at 25 °C under the assay conditions.

Results and discussion

Standard fed-batch cultures for aldolase production (in the present work rhamnulose-1-phosphate aldolase, RhuA) were performed as indicated in the "Materials and methods" section.

Their main characteristics were: (1) fed-batch growth using a predefined exponential profile; (2) pulse IPTG induction; and (3) after induction, once glucose was detected at levels around 0.5 g L^{-1}, feeding policy was interrupted and restarted according with off-line glucose analyses. This operational procedure avoids glucose accumulation, which could favor partial proteolysis of the target protein with loss in protein quality [17]. A modification of the above procedure was to use predictions of a mathematical model to anticipate glucose accumulation acting on the feeding flow rate. As previously reported [18], protein production was similar, with the added value of improving process automation and reducing nutritional requirements (35 % less nutrients).

The ratio inducer/biomass at the induction moment affects protein quality, measured in terms of activity units (U) per unit mass of RhuA, U mg^{-1}RhuA. Figure 1 presents this dependency. The actual selected value of 3 μmol IPTG g^{-1} DCW corresponds to a maximum of around 8.8 U mg^{-1}RhuA. Under the indicated conditions, standard cultures yielded an average of 950 U g^{-1}DCW and 108 mg RhuA g^{-1}DCW.

As pointed out in the Introduction section, knowledge of the system will be used to propose modifications at both operational and nutrient requirements level, with the aim of setting an operational procedure to be standardized and translated to higher scale production.

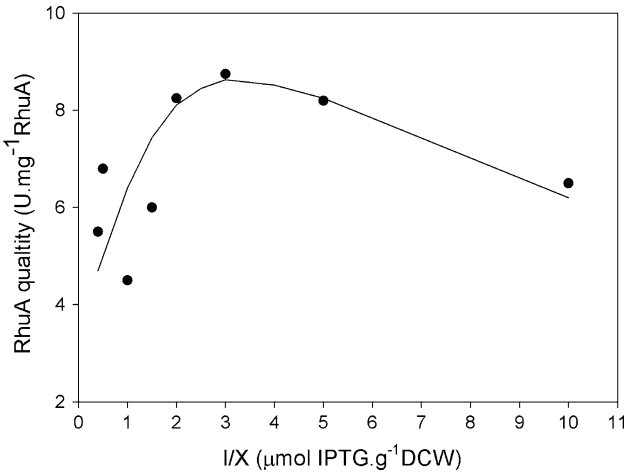

Fig. 1 Effect of the inducer-biomass ratio I/X (µml IPTG g^{-1} DCW) on RhuA aldolase quality (U mg^{-1}RhuA) in fed-batch standard cultures

Post-induction behavior

The analysis of post-induction cultures led to some conclusions about the applied feed policy during protein overexpression. The first one was observed when growth was analyzed both in terms of optical density and flow cytometry. Figure 2 shows an example of both measurements for non-induced stage (a) and induced stage (b). In the non-induced period, both measurements are coincident, meaning that OD increase is due to an increase in the number of cells. Nevertheless, after induction, cell concentration measured by flow cytometry is much lower than the value obtained from OD measurements. The same result was observed for all the cultures, indicating that the OD increase is not only due to changes in the number of cells, but also to other factors like changes in shape or other properties. One consequence of the above observation is that the nutritional requirements can be assumed to be different for induced and non-induced cells.

A second experimental finding can be seen in Fig. 3. After induction, specific growth rate, calculated from OD data, had an initial increase, although nutrients flow rate did not have substantial changes from the last moments of non-induced stage. Later on, there was a sustained decrease of specific growth rate during protein expression until growth arrest, and therefore nutritional requirements also changed.

In summary, an exponential feeding after induction implies an excess of nutrients, because the nutrients supply is not consistent with culture growth. As a result, glucose starts to accumulate and feeding must be stopped several times to maintain low glucose levels as desired. The alternate start and stop of feeding produces sudden changes in the cellular environment so that cells must readapt continuously, and is thus an additional factor for cellular stress during protein overproduction.

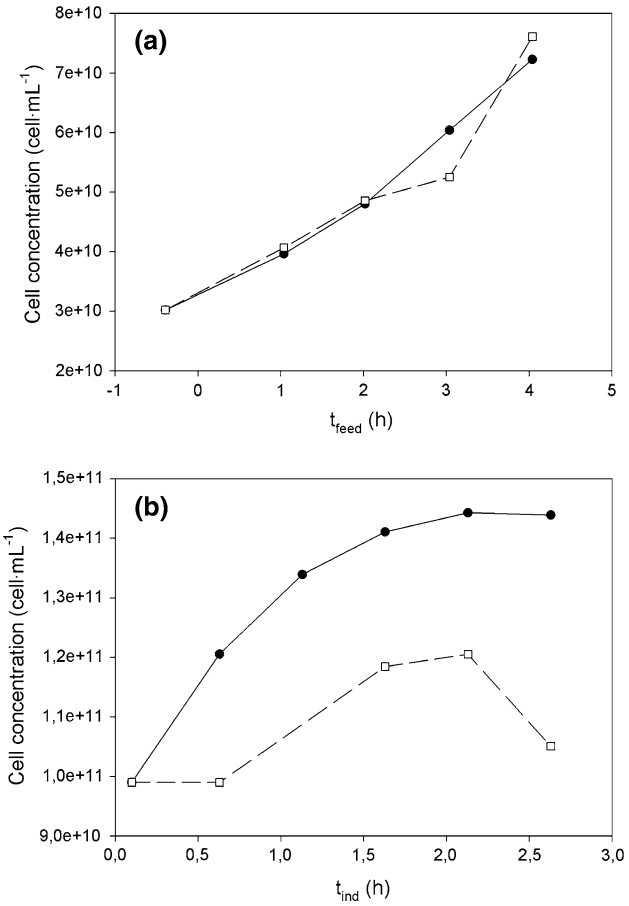

Fig. 2 Cell concentration from OD measurements (*filled circle*) and cell concentration from flow cytometry (*square*). (**a**) Evolution along feeding time for a non-induced culture; (**b**) Evolution with time after induction for culture induced at I/X = 3

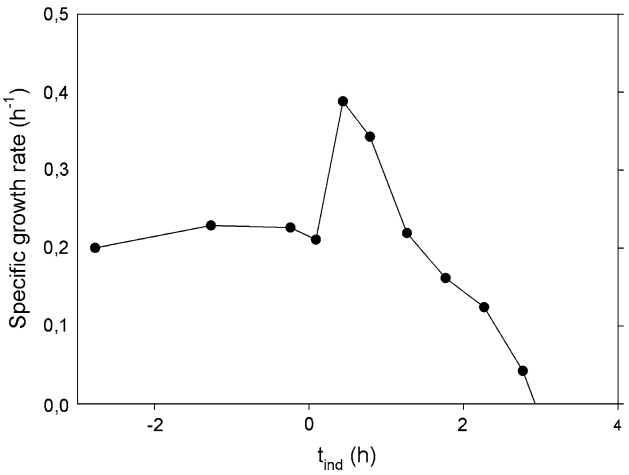

Fig. 3 Specific growth rate (µ) before and after induction for a standard fed batch culture

Alternative post-induction feeding policies

The proposed alternative for post-induction policy was to replace exponential addition profile by a constant flow rate. The aim is avoiding frequent feed stop-start sequences as a consequence of the control of glucose concentration bellow 0.5 g L^{-1} and to keep a more homogeneous environment for the cells.

At the end of the non-induction stage, the glucose fed per gram of dry cell weight and hour was calculated from the glucose flow rate and the biomass concentration according to the following equation:

$$F_{specific}\left(\frac{g_{gluc}}{g_{DCW}\cdot h}\right)=\frac{F_{gluc}\left(\frac{g_{gluc}}{h}\right)}{X\left(\frac{g_{DCW}}{L}\right)\cdot V(L)},$$

where $F_{specific}$ is the specific flow rate of glucose to be fed; F_{gluc} is the glucose flow rate at the end of the non-induced stage; and X and V are the dry cell weight concentration and the liquid volume, respectively, at the end of the non-induction stage.

The value for standard cultures was 0.55 g glucose g^{-1} DCW h^{-1}, and this was assumed, as a first approach, to be enough for protein overexpression and cell growth and maintenance during induction stage. Working with a feeding flow rate of 0.55 g glucose g^{-1} DCW h^{-1}, glucose did not start to accumulate until the end of the induction stage and after the maximum production of RhuA was attained, showing that this feeding rate was quite equilibrated between excess and defect of nutrients.

Four experiments were compared: (1) a standard experiment; (2) a model based experiment; (3) a constant feeding flow rate (0.55 g glucose g^{-1} DCW h^{-1}) culture; and (4) a constant feeding flow rate (0.27 g glucose g^{-1} DCW h^{-1}, 50 % of the value of experiment 3) culture.

Experiments 1–3 are different in the interruption frequency of the feed: model based control led to higher frequency of interruptions than standard operation, and the constant flow rate experiment has no interruptions of the feed. The fourth experiment is presumed to have a shortage of nutrients because of the low value of flow rate used.

The amount of RhuA produced per unit mass of DCW is presented in Fig. 4, as a percentage of the maximum obtained in a standard culture. It seems clear that reducing feed to one half (0.27 g glucose g^{-1} DCW h^{-1}) has a negative effect on protein production. Concerning the results of the other three experiments, it can be seen in Fig. 4 that the highest amount of RhuA produced is in the constant flow rate experiment (no interruptions of the feed), and that the lowest production is in the model based experiment (feeding more frequently interrupted). Standard culture showed a production in between, because feeding was not interrupted as frequently as in the model based culture.

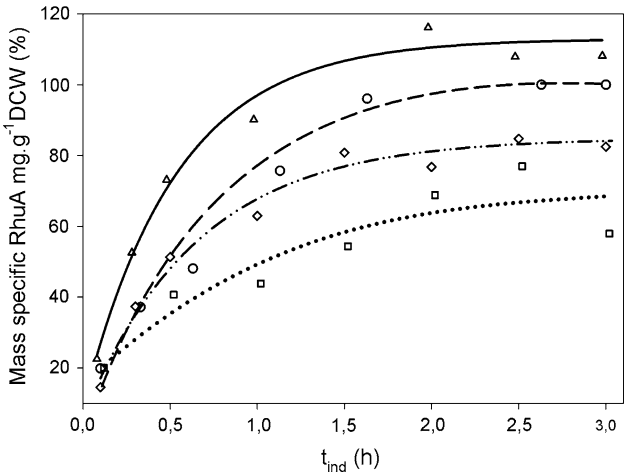

Fig. 4 Intracellular aldolase profiles for different feeding policies during induction phase expressed as percentage of maximum in a standard culture. (*circle*, *spaced hyphen*) standard culture; (*diamond*, *hyphen with dots*) model-based; (*triangle*, *solid line*) constant feeding at 0.55 g glucose g^{-1} DCW h^{-1}; (*square*, *dots*)constant feeding at 0.27 g glucose g^{-1}DCW h^{-1}

This is a result in favor of the hypothesis that cell environment is more constant when constant feeding is used, reducing the stress of the cell and yielding a higher production of RhuA per unit mass of DCW. Chen et al. [3] reported a similar behavior for glucosamine synthase production.

Amino acid supplementation for protein quality improvement

As pointed out in Introduction section, the high amino acid demand during protein overexpression constitutes a metabolic overload. The coordinated addition of amino acids during induction phase, based in a selection of the candidate amino acids from the target protein composition, has been suggested as an effective tool to alleviate cellular stress [9, 14, 15]. In the previous section, it was shown that maintaining medium homogeneity for a long time had a positive effect on aldolase quantity. In order to improve the enzyme quality (Units per mg of aldolase), medium supplementation with required essential amino acids was explored.

The amino acid composition of a subunit of the rhamnulose-1-phosphate aldolase tetramer was obtained from NCBI (National Center for Biotechnology Information) data bank [12]. Figure 5 shows the relative frequency of each amino acid in the primary structure of the aldolase, together with the mean *E. coli* cytoplasmic proteins composition [1].

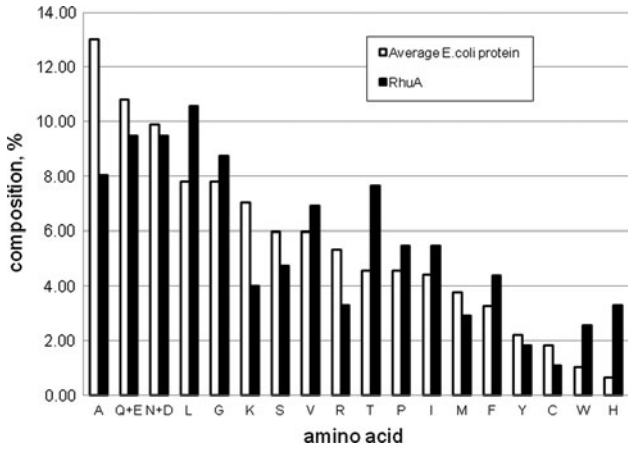

Fig. 5 Amino acid relative frequency (%) in the primary structure of RhuA and the average cytoplasmic proteins of *E. coli*

Table 1 Amino acid supplemented cultures: Aldolase quality, amount and specific activity expressed as percentage of maximum in a standard culture

	Protein quality U mg^{-1} RhuA (%)	RhuA amount mg RhuA g^{-1} DCW (%)	Specific activity U g^{-1} DCW (%)
Standard culture	100	100	100
Histidine addition	80	150	105
Leucine addition	130	105	135
Threonine addition	110	130	125
Triptophane addition	120	105	115
Glycine addition	80	140	120

The selected amino acids for supplementation were histidine (H), tryptophan (W), threonine (T), due to the biggest differences in relative amount; leucine (L), highly present in RhuA, and glycine (G), due to auxotrophy. According to the literature [14], the amount of every amino acid to be added was calculated to be 20 % of that necessary for the synthesis of the expected aldolase in a standard culture (around 3 g of protein). This 20 % is estimated with the aim of avoiding possible inhibitions in the amino acids synthetic pathway. Supplementation was performed in fed-batch cultures just before induction. Combinations of the above (or other) amino acids could also be investigated.

The obtained results are summarized in Table 1. Final biomass concentration was very similar in all cases. The highest quality of protein as activity units per unit mass of RhuA was obtained for the leucine addition case. Leucine improved the amount only slightly, 5 %, but the combination of amount and quality, 30 %, yielded the highest specific activity, 35 % more than standard culture.

Pilot plant operation

From the above experiments, it can be seen that a more constant cell environment led to an improvement of the amount of protein produced per g DCW, and that the addition of leucine led to a better quality of the protein than in the standard cultures. These aspects should be taken into account to improve the production processes, mainly at larger scales, such as pilot and industrial.

Aldolase production was translated to a pilot scale (50 L fermentor), operating in fed-batch mode as indicated in "Materials and methods". The operational strategy was based on all the above-presented results.

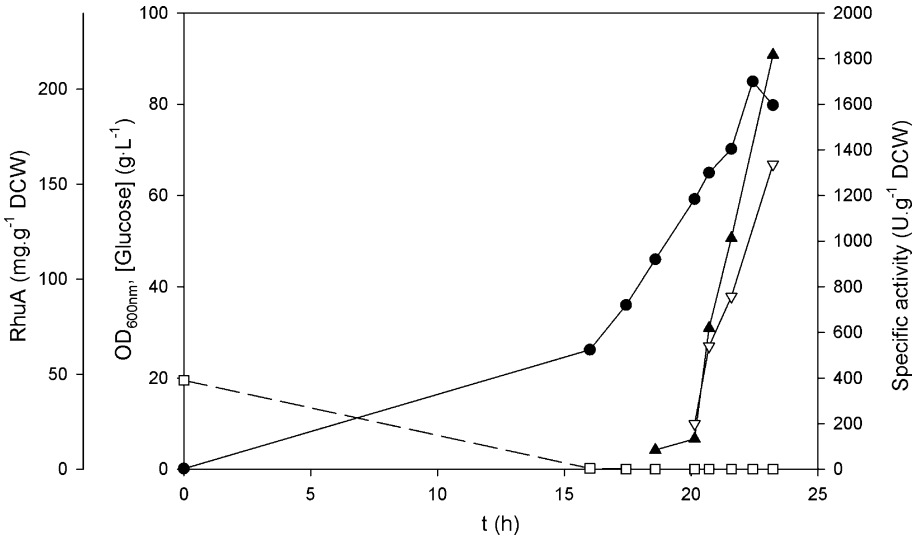

Fig. 6 Time profiles for the pilot plant RhuA production. The dashed arrow indicates the induction moment. (*filled circle*) Optical density; (*square*) Glucose concentration; (*filled triangle*) Specific activity; (*inverted triangle*) RhuA mass specific amount

Consequently, before induction, leucine was added at a concentration of 70 mg L^{-1}. Moreover, an approximately constant feeding profile was implemented after induction. In this case, it was between 0.5 and 0.4 g glucose g^{-1} DCW h^{-1}, slightly lower than at laboratory scale, in order to prevent possible glucose accumulation at the end of the induction stage.

The time profiles of biomass, glucose and aldolase concentration and aldolase activity are presented in Fig. 6. Biomass growth was maintained until 3.5 h after induction without glucose accumulation, and reached biomass concentrations similar to those in standard laboratory experiments. On the other hand, specific production of RhuA reached high levels in both mass and activity units: 160 mg RhuA g^{-1} DCW and 1800 U g^{-1}DCW, which means a quality of 11.3 U mg $RhuA^{-1}$.

The results can be better understood if the pilot plant behavior is compared with the different production strategies: standard culture, model-based feeding, and leucine addition. These are presented in Fig. 7a, b, c. Quality of the protein enhanced as a consequence of leucine addition. The obtained levels, 11.3 U mg^{-1} RhuA, are similar to the laboratory culture with leucine addition (Fig. 7a). This is an improvement of 30 % with respect to standard operation. On the other hand, nutrient addition at an almost constant rate maintained a more homogeneous environment for the cells, thus allowing for higher protein amount per g DCW (Fig. 7b). The 50 % increase over standard culture is higher than the 20–25 % observed at laboratory scale for constant feeding. To explain this fact, one can hypothesize about the differences in feeding system. Feeding was performed employing a programmable pump in the case of the pilot plant, which could ensure a more constant flow (and thus, homogeneous environment for the cells) than the microburette used at the laboratory scale, with alternate charge and feed steps. Some other factors could also be taken into account. Although the global homogeneity was proven to be good in previous studies, some local gradients may exist, influencing the behavior of the system.

The combination of both factors gives the activity per g of DCW presented in Fig. 7c. A 100 % increase of specific activity (U g^{-1} DCW) over the standard culture was obtained in pilot plant operation.

From the global perspective of the process, the integrity of the protein produced (maintenance of the six-histidine tag) was tested by submitting cell lysate to metal-chelate affinity chromatography, as explained in the "Materials and methods". RhuA was almost totally attached to the support, giving recovery yields higher than 90 %. This yield allows both recovery of the produced aldolase, as well as its direct use as immobilized biocatalyst.

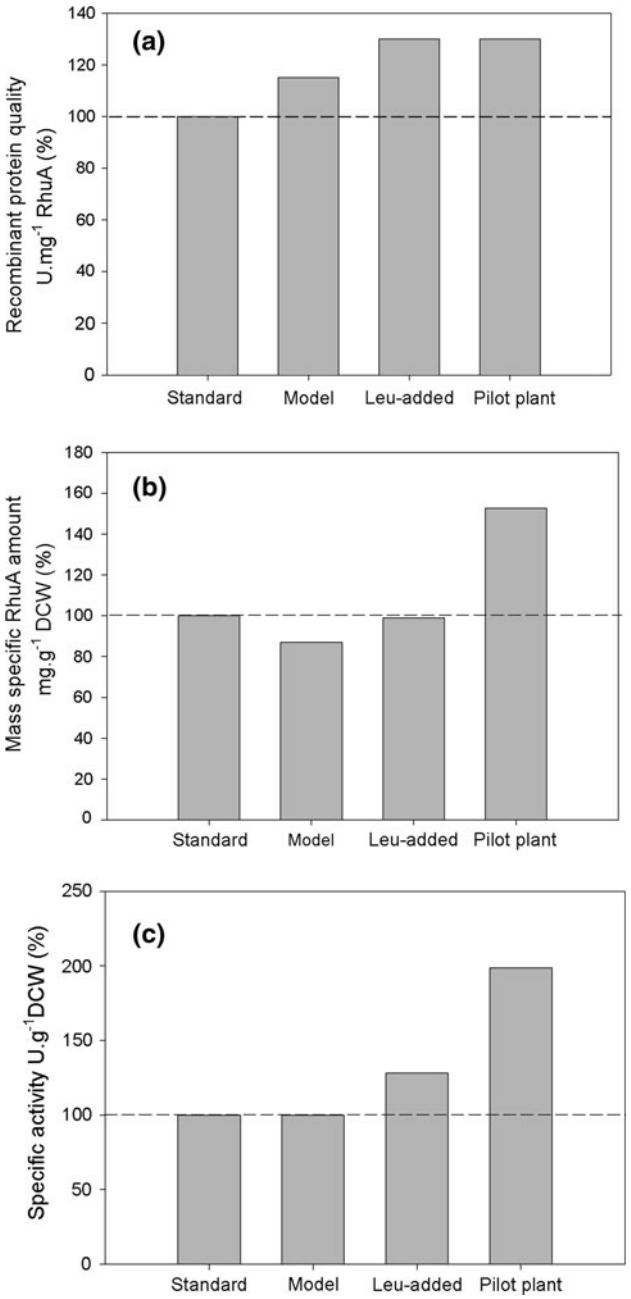

Fig. 7 Performance of the different operational strategies and the pilot plant operation in terms of (**a**) protein quality, (**b**) mass RhuA amount and (**c**) specific activity

Conclusions

In the present work, different factors affecting recombinant aldolase production in *E. coli* fed-batch cultures have been assessed.

The ratio of the inducer concentration to the biomass concentration affected the quality of the protein produced. In the case of this study, a value around 3 μmol of IPTG per g of DCW gave the best results.

It was experimentally noted that the increases in OD after induction were the result of an increase in number of cells and changes in cell morphology. This finding, together with the decrease of specific growth rate calculated from OD measurements, indicated that exponential feeding after induction was not the best operational strategy. A more constant feeding profile after induction has been demonstrated to have a favorable effect on the amount of aldolase produced per unit mass of DCW. Temporal homogenization of the cell environment seems to be the reason for less cellular stress leading to higher protein yields.

On the other hand, among the amino acids studied for supplementation before induction, leucine addition was shown to positively affect the quality of the protein as activity units per unit mass of RhuA.

The combination of both improvements was employed to design a proper operational procedure for pilot plant production. In this case, the obtained protein was of the highest quality, 11.3 U mg $RhuA^{-1}$ with a production of 45000 U L^{-1}, double that of the standard one. The obtained aldolase has been almost quantitatively purified (> 90 %) by affinity chromatography.

The principles and methodologies developed here could be extended to the production of other proteins employing similar expression systems.

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