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ORIGINAL PAPER

Alternative Heterologous Expression of L-Arabinose Isomerase from *Enterococcus faecium* DBFIQ E36 By Residual Whey Lactose Induction

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Abstract

This study reports an alternative strategy for the expression of a recombinant L-AI from *Enterococcus faecium* DBFIQ E36 by auto-induction using glucose and glycerol as carbon sources and residual whey lactose as inducer agent. Commercial lactose and isopropyl *β*-D-1-thiogalactopyranoside (IPTG) were also evaluated as inducers for comparison of enzyme expression levels. The enzymatic extracts were purified by affinity chromatography, characterized, and applied in the bioconversion of D-galactose into D-tagatose. L-AI presented a catalytic activity of 1.67 ± 0.14, 1.52 ± 0.01, and 0.7 ± 0.04 U/mL, when expressed using commercial lactose, lactose from whey, and IPTG, respectively. Higher activities could be obtained by changing the protocol of enzyme extraction and, for instance, the enzymatic extract produced with whey presented a catalytic activity of 3.8 U/mL. The specific activity of the enzyme extracts produced using lactose (commercial or residual whey) after enzyme purification was also higher when compared to the enzyme expressed with IPTG. Best results were achieved when enzyme expression was conducted using 4 g/L of residual whey lactose for 11 h. These results proved the efficacy of an alternative and economic protocol for the effective expression of a recombinant L-AI aiming its high-scale production.

Keywords Cheese whey · L-Arabinose isomerase · *Escherichia coli* expression · D-Tagatose · Auto-induction

Introduction

The pursuit of healthy eating habits has led to a progressive generation of functional food products that contribute to a general state of health for those who consume them [1]. In this context, the consumption of substances that can influence the physiological functions both related to disease prevention and associated with health benefits, such as nutraceuticals [1], has gained attention.

Among many nutraceuticals available on the market, D-tagatose is a ketohexose that stands out for its high sweetness power (90%) versus its low caloric value when

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compared to D-sucrose [1–3]. There are many studies that present the wide range of benefits associated with the use of D-tagatose. Ingestion of D-tagatose, for example, does not raise the glycemic index [2, 4] being also indicated for treatment of metabolic syndrome [5]. Furthermore, D-tagatose exhibits antidiabetic [5, 6], antioxidants [1], prebiotics [7], and non-cariogenic properties [4].

D-Tagatose can be produced either by chemical or biological processes [8]. However, the biological production of D-tagatose has several advantages, such as, the use of mild reaction conditions and high specificity due to the use of L-arabinose isomerase (EC 5.3.1.4, L-AI), which isomerizes D-galactose into D-tagatose [9–11]. L-AI can be obtained from various bacterial strains, such as *Enterococcus faecium* [12], *Pediococcus pentosaceus* [13], *Saccharomyces cerevisiae* [14], *Thermoanaerobacterium saccharolyticum* [15], *Geobacillus stearothermophilus* [16], *Pseudoalteromonas haloplanktis* [17], among other sources of origin.

Heterologous expression of L-AI using *Escherichia coli* as host is usually done by the use of isopropyl-1-thio-*β*-D-galactopyranoside (IPTG), a synthetic lactose analog [18], as an inducer agent which was applied in many studies [12, 13, 15, 19]. This inducer acts by removing the repressor protein present in the *lac operon*, promoting enzyme expression [20–22]. However, this inducer demands a high cost being also cytotoxic [23, 24], in addition to generating low production yields when compared to auto-induction systems [18, 22]. Auto-induction was proposed by Studier [22] and has been explored since for the expression of different recombinant proteins. The method is based on the regulation of *lac operon* under diauxic growth conditions in glucose, glycerol, and lactose mixtures. *E. coli* preferably consumes glucose during the early stages of growth. When this carbon source is exhausted, lactose and glycerol sustain the later stages of growth and production of recombinant proteins [25]. The presence of glucose in the initial stage ensures that protein expression is low, due to catabolite repression. When glucose is consumed, catabolic repression ceases, allowing the consumption of lactose and glycerol. Lactose intake results in the formation of allolactose by a promiscuous reaction catalyzed by *β*-galactosidase. This disaccharide will be the physiological inducer of the *lac operon* [25], initiating the production of the recombinant enzyme. In this direction, the auto-induction (AI) method is considered to be economical and simple, allowing high expression of protein simultaneously avoiding cytotoxicity [20, 22, 26].

In order to allow industrial production of D-tagatose, it is also important to develop a process for obtaining L-AI at large scale. L-AI production could be achieved by heterologous expressions in a low-cost production medium using renewable substrates and/or industrial by-products. Whey is the liquid part that is formed after coagulation of casein present in milk when cheese is produced. Milk consists of approximately 85–95% of whey by volume, and whey contains 55% of the nutrients found in milk [27], especially lactose, which corresponds to 60–80% of total solids [28]. Furthermore, cheese whey is considered a waste pollutant derived from the dairy industry [18] and, therefore, a low-cost substrate [29].

Therefore, in this work, a possible replacement of IPTG as inducer agent was investigated and a promising method for the heterologous expression of *Enterococcus faecium* L-AI was proposed. This enzyme has shown relevant physicochemical properties with industrial feasibility [12]. In this method, IPTG is replaced by residual whey lactose through an auto-induction process (AI).

To the best of our knowledge, this protocol is innovative for L-AI expression and the present report is the first to report auto-induction for this enzyme.

Materials and Methods

Bacterial Strain and Construction of the Recombinant L-AI

Escherichia coli BL21 (DE3) was transformed with the vector harboring the recombinant *araA* gene of *Enterococcus faecium* DBFIQ E36 [30] by Manzo et al. [12], and enzyme production was induced through the *lac operon*. The recombinant protein has 6 histidine residues (His) in the N-terminal portion of its structure, a molecular weight around 55.87 kDa and an isoelectric point of 5.07 [12].

Conservation and Reactivation of Recombinant Culture

Stock cultures of transformed *E. coli* BL21 cells were maintained in Luria Bertani medium containing 100 µg/mL of ampicillin (LB_{amp}) with 20% (*v*/*v*) glycerol as cryoprotect agent and preserved at − 80 °C for ensuring their stability. Reactivation cultures were done by adding 0.1 mL of preservation culture to LB_{amp} medium and incubated overnight at 37 °C.

Culture Medium and Assay Conditions

For the expression of rL-AI, enzyme production was studied using both induction and auto-induction protocols employing three different inducers: IPTG, commercial lactose and residual whey lactose. Expression of the protein of interest by auto-induction was conducted using the methodology proposed by Studier [22].

In the first experiments, in order to prove the effective production of L-AI, enzyme production was induced with 0.5 mM of IPTG final concentration as inducer agent. A

volume of 2 mL of reactivated culture, 1% (*v*/*v*) inoculum, was transferred to 200 mL of LB_{amp} and incubated at 37 °C, 200 rpm until it reached to an $OD_{600\ nm}$ from 0.8 to 1.0. Immediately, the culture was induced for L-AI expression by the addition of IPTG during 4 h.

The auto-induction medium (ZYM-5052) [22], as seen in Table 1, was prepared using commercial lactose (Dinâmica—Química Contemporânea Ltda, Indaiatuba—SP-Brazil) as carbon source and induction, supplemented with ampicillin (100 µg/mL). In addition, 0.02% (*v*/*v*) of trace elements solution was added to the medium, and the pH was 7.0. The trace element solution was composed of 50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 2 mM $CoCl_2$, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM Na_2MoO_4, 2 mM Na_2SeO_3, and 2 mM H_3BO_3, which was sterilized by filtration using a 0.22 µm pore diameter filter. Then, 2 mL of pre-inoculum culture, with an $OD_{600\ nm}$ from 1.6 to 1.7, was added to 200 mL of culture medium and flasks were incubated at 37 °C and 200 rpm for 9–11 h.

For the use of lactose contained in powder whey (Alibra Ingredientes Ltda, Campinas-SP-BR) in auto-induction experiments, the solid by-product was diluted in water to a final concentration of lactose of 2 g/L, which was the lactose concentration in the medium ZYM-5052 proposed in the literature [22]. In order to evaluate the effect in enzyme production and since whey contains impurities and other nutrients, other concentrations of lactose were also investigated (4 or 8 g/L). Besides, due to the high amounts of protein in whey [31], an initial deproteinization treatment was conducted according to a previously described protocol [32]. After protein removal, the solution was supplemented with ZYM-5052 medium components and was sterilized at 110 °C for 10 min. Afterward, 0.02% (*v*/*v*) of trace elements solution was added to the medium. Finally, a volume of 2 mL of propagation culture, with an $OD_{600\ nm}$ from 1.6 to 1.7, was added to 200 mL of culture medium and flasks were incubated at 37 °C and 200 rpm for 9–11 h.

At different time lapses, culture samples (2 mL) were withdrawn by triplicate At the end of assay, cultures were centrifuged at 7000×*g* at 4 °C for 15 min. Supernatant was then stored for carbon source determination assays, and cells were subsequently washed with 0.1 M NaCl solution followed by centrifugation at 7000×*g* at 4 °C for 40 min. Cell pellets were then stored at − 20 °C for further cell growth analysis, protein expression studies, and enzyme activity assays.

All studies were conducted in triplicate and results were presented as the average of these values with their corresponding standard deviation.

Table 1 Composition of the auto-induction medium ZYM-5052 [22]

Component	Concentration
Triptone	1%
Yeast extract	0.5%
Na_2HPO_4	25 mM
KH_2PO_4	25 mM
NH_4Cl	50 mM
Na_2SO_4	5 mM
Glycerol	0.5%
Glucose	0.05%
Lactose	0.2%
$MgSO_4$	2 mM

Cell Lysis and Extraction of rL-AI

Cell pellets were resuspended in 50 mM Tris HCl pH 8.0 and 150 mM NaCl and cell lysis were conducted by sonication (Sonicator Qsonic, Q500 Sonicators, USA) at 30% of amplitude, for 4 min (working time and interval of 3 s) at 4 °C. Cell suspension was sonicated until a transparent pale yellow color was obtained. After cell disruption, samples were centrifuged (5000 rpm, 4 °C for 30 min) and supernatant (crude L-AI extract) was stored at − 20 °C and further used to determine enzyme activity, protein concentration, and perform some physicochemical characterization studies.

Enzymatic Activity Assay

For assessing the catalytic activity, a reaction mixture containing 0.4 mL of 625 mM D-galactose in 50 mM sodium acetate buffer pH 5.6 supplemented with 1 mM $MnCl_2$ and 0.1 mL of rL-AI extract was prepared. Afterwards, the mixture was incubated at 50 °C for 60 min and D-tagatose was determined by using the cysteine–carbazole–sulfuric acid method at 560 nm [33] and compared to a standard calibration curve prepared using D-tagatose. One unit of L-AI activity (U) was defined as the amount of enzyme capable of producing 1 µmol of D-tagatose per min at pH 5.6 and 50 °C.

Protein Quantification

Protein concentration was determined by the Bradford method [34] using bovine serum albumin as standard for calibration curve construction.

Analysis of rL-AI Expression Level By Tricine–SDS–PAGE

Protein expression levels during cultivation were determined through time by Tricine–SDS–PAGE electrophoresis and employing Coomassie Brilliant Blue R-250 as staining agent [35]. Briefly, crude extracts and *pellets,* were weighted (0.01 g of *pellet*) and resuspended into 1 mL of sample loading buffer, with a final concentration of 12% (*w*/*w*) glycerol, 4% (*w*/*w*) SDS, 2% (*v*/*v*) *β*-mercaptoethanol, 0.01% (*w*/*v*)

Coomassie Blue G 250 and 0.05 M Tris HCL, pH 6.8. A wide range molecular weight marker (GE Healthcare Life Sciences, USA), from 11 to 245 kDa, was employed as protein standard for rL-AI molecular weight estimation.

Protein expression patterns were calculated through the measure of the gel bands density by the use of the scanned electropherograms with the image processing software ImageJ. Values were informed as relative percentages in terms of the highest area value achieved in each gel. No changes were made to the images other than cut, paste, and resize [18].

Cultivation Parameters

Substrate conversion was calculated according to Fogler [36] as shown in Eq. (1):

$$x_i = \frac{S_{i0} - S_i}{S_{i0}} \times 100 \quad (1)$$

where S_0 refers to the initial substrate concentration, S refers to the sample substrate concentration taken at each time interval and i corresponds to the employed carbon sources (glucose, glycerol, and lactose).

The volumetric productivity (P_P) was calculated as the ratio of the enzyme activity to the cultivation time (t).

Specific growth rates (μ_X) were estimated by plotting the data for cell concentration (X) versus time (t), at the exponential growth phase, and using Eq. (2):

$$\mu_X t = \ln\left(\frac{X_i}{X_0}\right) \quad (2)$$

Enzyme Purification

Purification of crude enzyme extracts was performed by immobilized metal affinity chromatography (IMAC) using Ni-Sepharose 6 Fast Flow matrix with a degree of activation of 15 µmol of Ni^{2+} ion per ml of wet resin (GE Healthcare). Chromatography column (1.5 cm high per 1.5 cm wide; volume column: 3 mL) was equilibrated with five column volumes of 50 mM Tris HCl pH 8.0 and 150 mM NaCl buffer at a flow rate of 1.5 mL/min which was maintained constant throughout the experience. Then, 12 mL of enzymatic extract with a protein concentration of 7.1 mg/mL was applied to column. Non-retained proteins were recovered and immediately washed with 50 mM Tris–HCl pH 8.0, 150 mM NaCl, and 6 mM imidazole buffer in order to ensure the removal of the weakly bound proteins. After that, five column volumes of elution buffer (50 mM Tris HCl pH 8.0, 500 mM NaCl and 250 mM imidazole) was applied to the column in order to collect the purified protein. Finally, purified rL-AI was dialyzed for 24 h against 50 mM Tris–HCl pH 8.0 for imidazole remotion and further analyzed by Tricine–SDS–PAGE.

Quantification of Carbon Sources

The concentration of glucose, glycerol and lactose (both commercial and from whey) present in the ZYM-5052 auto-induction culture medium, were determined by High Efficiency Liquid Chromatography (HPLC) equipped with a refractive index detector (Waters, Milford, MA, USA). During the production of the enzyme of interest, aliquots were withdrawn from the process. All samples were syringe filtrated with a 0.45 µm pore diameter filter and a volume of 20 µL of each sample was injected into a HPX-87H column (Bio-Rad, Hercules, CA, USA). Chromatography conditions were set at a flow rate of 0.5 mL/min at 65 °C for 20 min and employing an isocratic gradient of 5 mM sulfuric acid as mobile phase.

Effect of pH and Temperature on rL-AI Activity

The effects of pH on the purified recombinant L-AI arabinose isomerase enzymatic activity was determined using 50 mM sodium acetate buffer, for pH 4.0, 5.0, and 5.6, 50 mM sodium phosphate buffer, for pH 7.0 and 8.0, and 50 mM sodium bicarbonate buffer, for pH 10.0. All buffers were supplemented with 1 mM $MnCl_2$ final concentration.

The effect of temperature was investigated at 40, 50, 55, 60, and 65 °C. For that, enzyme was assayed for 60 min, maintaining the reaction ratio of 0.4 mL of D-galactose (625 mM) solution and 0.1 mL of enzymatic solution (1.8 µM), achieving a 500 mM D-galactose final concentration.

D-Tagatose Bioconversion By the Purified L-AI

Solutions containing 0.1 mL of purified enzymatic extract (1.8 µM, 0.114 U/mL) and 0.4 mL of D-galactose were prepared in 50 mM sodium acetate buffer (pH 5.6 supplemented with 1 mM $MnCl_2$) and incubated at 55 °C. Two different final D-galactose concentrations were used, 164 and 500 mM. Aliquots were withdrawn at defined time intervals up to 52 h. D-Tagatose produced was determined using the cysteine–sulfuric acid–carbazole colorimetric method [33].

Isomerization yield was calculated as the ratio between the concentrations of D-tagatose (product) and the initial concentration of D-galactose (substrate).

Results and Discussion

Production of rL-AI Using An Auto-Induction Culture Medium Containing D-Lactose

One of the main challenges when pursuing higher enzyme concentrations is the proper design of a culture medium in terms of the optimization of the composition and/or concentrations of their nutrients. Therefore, exploratory experiments were conducted in shake flasks to test the ability of using an auto-induction medium containing D-lactose for L-AI production. For comparison, expression of L-AI was also done in LB_{amp} medium with the addition of 0.5 mM of IPTG as inducer agent for 4 h [12]. Then, the substitution of IPTG by lactose or solutions containing lactose (deproteinized whey) was investigated and the production of the target protein was followed by gel electrophoresis as shown in Figs. 1 and 2.

Cultures were carried out with three different residual whey lactose concentrations (2, 4 and 8 g/L) and the best expression pattern was achieved using 4 g/L at 11 h (Table 2).

Cells responded slower to residual whey lactose induction (11 h) when compared to the commercial lactose (9 h). Also, enzyme production with commercial lactose was higher than those obtained with residual whey lactose (Table 2). When comparing the enzymatic activity, it was observed that commercial lactose and residual whey lactose were better inducers than IPTG (Table 3). In the same direction, enzyme activities when using lactose or whey lactose instead of IPTG were twofold higher. Auto-induction protocols have other advantages besides the higher expression. For instance, the use of lactose is cheaper and is added at the beginning of the bioprocess [22], while the use of IPTG usually demands a high cost and must be added into the culture medium at late exponential growth phase [20]. Additionally, productivity of the product (P_P) was higher, not only in the processes which employed commercial lactose but also in that which whey lactose was used when compared to the use of IPTG as inducer agent. This is clearly a desirable behavior for these culture media to be employed in industrial processes directed to L-arabinose isomerase production.

It is important to emphasize that, at first, only one cell disruption cycle was performed for enzyme release. However, seeking higher extract yields and, consequently, a higher concentration of the protein of interest, cell suspension was submitted to three consecutive lytic cycles. This data are also shown in Table 3.

Time Course for Cell Growth and L-Arabinose Isomerase Production

Batch cultivations were conducted aiming to measure both cell growth and substrate consumption during rL-AI expression. Figure 3 shows the obtained cell growth profiles in

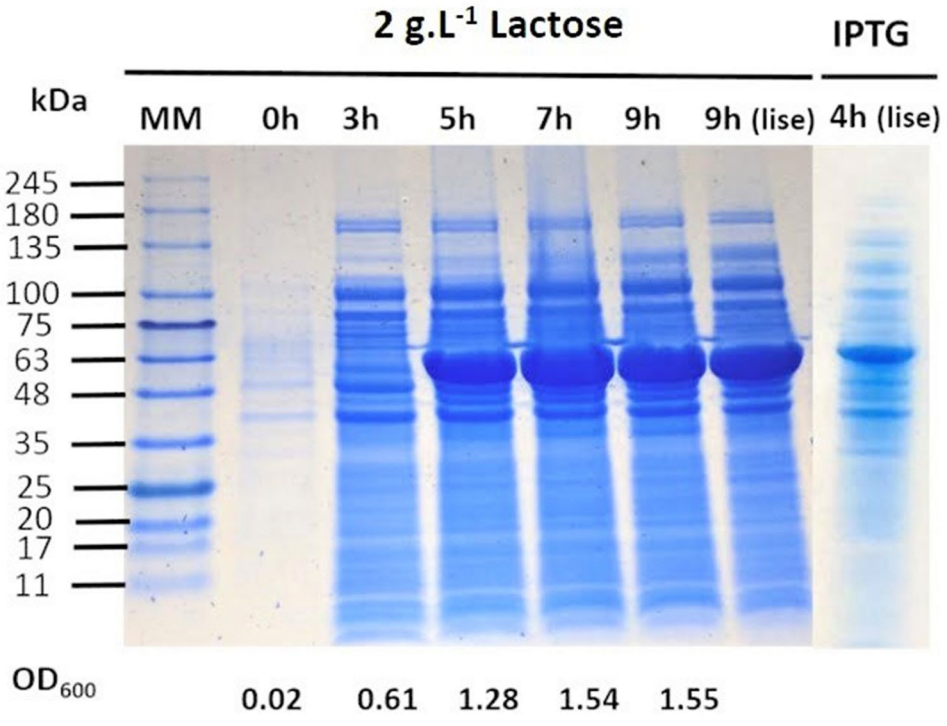

Fig. 1 Electrophoretic profile of cell extracts produced after *E. faecium* L-AI overexpression in *E. coli* BL21 using auto-induction medium with commercial lactose or IPTG: effect of type of inducer and time of induction. Molecular weight standards (MM) are on the left side of the gel and the sample time is given above each lane

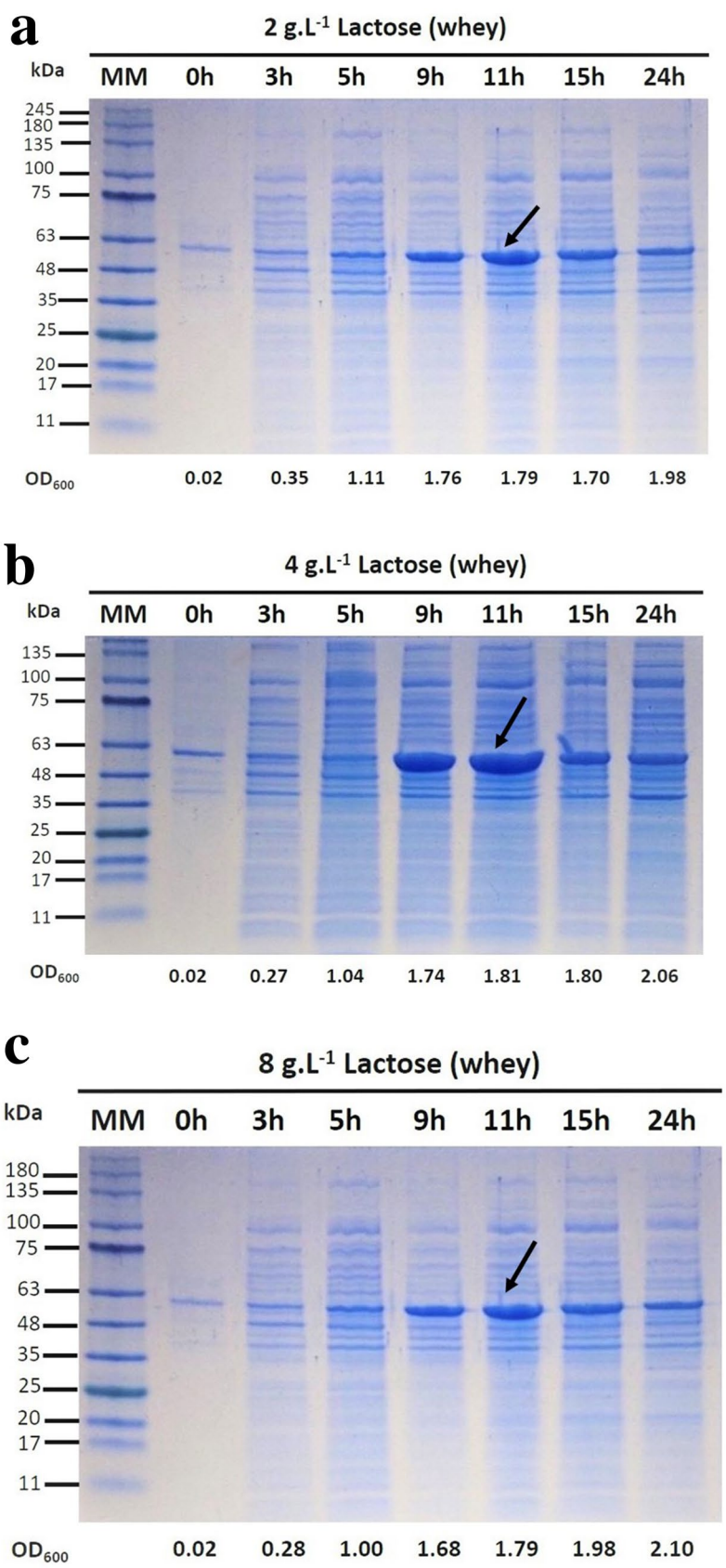

Fig. 2 Expression profiles of L-arabinose isomerase using different concentrations of residual whey lactose (whey) as the inducer. Induction was done at the concentration of 2 g/L (**a**), 4 g/L (**b**), and 8 g/L (**c**). Target recombinant protein is indicated by *arrows*

Table 2 Area of the rL-AI bands and relative percentage (%) of protein production using an auto-induction medium containing commercial lactose or residual whey lactose for induction

Sampling time (h)	Lactose (commercial) 2 g/L		Lactose (whey) 2 g/L		Lactose (whey) 4 g/L		Lactose (whey) 8 g/L	
	Area	*RP* (%)	Area	*RP* (%)	Area	*RP* (%)	Area	*RP* (%)
3	0	0	1,380,698	32	1,280,870	18	1,201,577	23
5	9,836,217	81	1,825,941	43	1,281,234	18	1,938,012	38
9	12,117,246	100	4,006,891	93	6,767,125	96	4,360,184	85
11	n.d.	–	4,295,154	100	7,017,196	100	5,113,690	100
15	n.d.	–	3,693,719	86	2,636,790	38	3,664,548	72
24	n.d.	–	2,517,941	59	2,787,033	40	2,217,577	43

Band areas were calculated with ImageJ and *RP* (%) was calculated using the highest value of samples applied in the same gel

n.d. not determined

Table 3 Shake flasks production of rL-AI by *E. coli* expressing L-AI from *E. faecium* DBFIQ E36 using different inducers: IPTG, commercial lactose (lac), and residual whey lactose (lac–whey).

CM	Ind	*T* (h)	*At* (U/mL)	*Pr* (mg/mL)	*Sp* act (U/mg)	P_P (U/mL/h)
LB	IPTG	8	0.7 ± 0.04	7.40 ± 0.2	0.10	0.09
ZYM-5052	Lac	9	1.67 ± 0.14	2.68 ± 0.1	0.62	0.18
ZYM-5052	Lac–whey	11	1.52 ± 0.01	4.60 ± 0.1	0.33	0.14
ZYM-5052	Lac–whey*	11	3.8 ± 0.00	7.08 ± 0.06	0.53	0.34

CM culture medium, *Ind* inducer, *t* time, *At* enzymatic activity, *Pr* protein concentration, *sp act* specific activity, P_P productivity, *Lac* commercial lactose, *Lac-Whey* residual whey lactose, *Lac-Whey** residual whey lactose, three lysate cycles

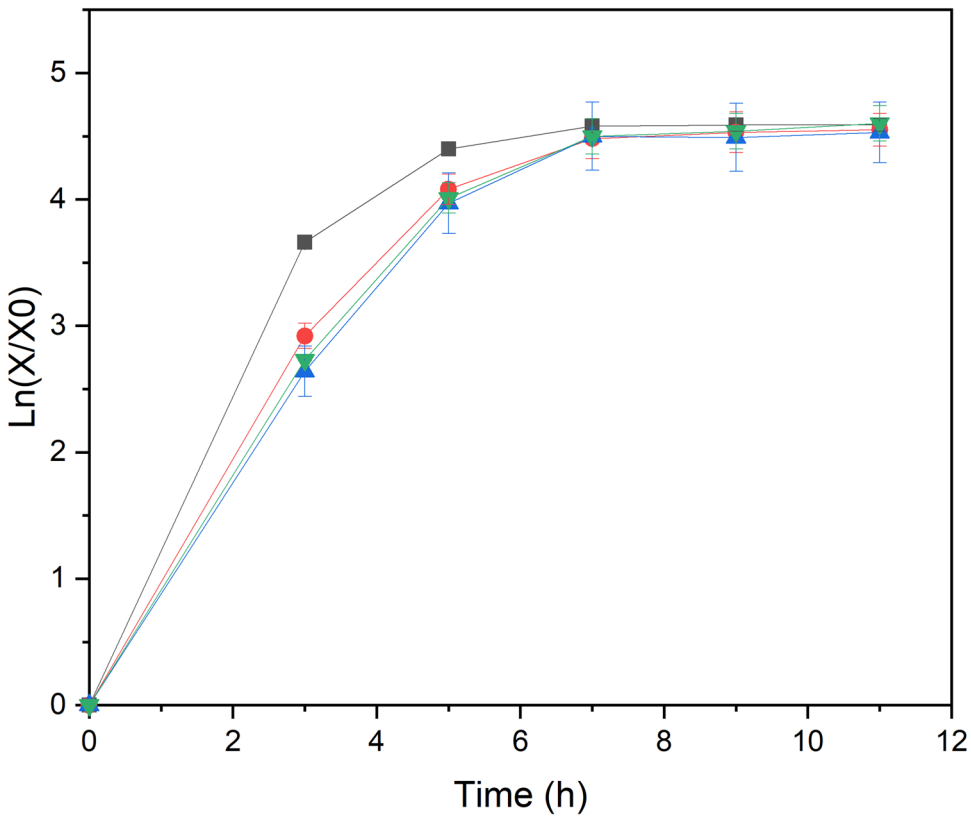

Fig. 3 Effect of different inducers on recombinant *E. coli* growth expression. Commercial lactose (*filled black square*), lactose 2 g/L (whey) (*filled red circle*), lactose 4 g/L (whey) (*filled blue triangle*), and lactose 8 g/L (whey) (*filled inverted green triangle*) (Color figure online)

auto-induction culture media using both commercial lactose and residual whey lactose (2, 4, and 8 g/L).

From the data shown in Fig. 3, cell growth rates (μ_X) were estimated. In this sense, a specific growth rate (μ) of 0.907/h was obtained when commercial lactose was used, whereas μ_X ranged from 0.801 to 0.827/h for residual whey lactose. Although the specific growth rate was the highest when using pure lactose, this difference was not significant, not compromising the enzyme production process since same cell concentrations were achieved after 9 h of cultivation. Furthermore, productivities were higher when whey lactose was used, as seen in Table 3.

Cell growth and enzyme production may be affected by several factors such as the type and level of available nutrients. ZYM-5052 culture medium used in this work is composed of three carbon sources including the inducer agent: glucose, glycerol, and lactose [22]. Figure 4a, b show the conversion (see Eq. 1) of the three carbon sources during L-AI production using commercial lactose and residual whey lactose as inducers.

Glucose was consumed first and faster than the other carbon sources. Hence, it was totally depleted before 5 or 3 h of process duration for the trials when commercial lactose or residual whey lactose were used, respectively. It is important to notice that glucose was consumed for cell growth and when it was exhausted, stationary phase was reached (Fig. 3). On the other hand, during this period of time, lactose and glycerol concentration remained almost constant, for both cases.

Upon exhaustion of glucose, glycerol and lactose consumption started (Fig. 4a, b). When using commercial lactose, both of them were initially assimilated simultaneously, but from 9 h, glycerol conversion remained constant (around 20%), while lactose conversion increased with time up to 77%. This behavior was also evidenced when comparing the volumetric rate of glycerol and lactose consumption profiles, as shown in Fig. 5a. It was possible to see that glycerol consumption rate decreased, while those for lactose remained constant for a time period higher than 2 h. Furthermore, we have observed that the beginning of consumption for both glycerol and lactose was around 3 h of fermentation time, overlapping with the beginning of the cell growth exponential phase. At 5 h of assay, the volumetric consumption rate was maximal for glycerol and lactose.

When residual whey lactose was used (Fig. 4b), at the end of cultivation time, only 27.7% of the available glycerol was used, reaffirming that this was a secondary carbon source that did not produce catabolic repression. In both cases, when using commercial lactose or residual whey lactose, it was possible to see that lactose consumption promoted glycerol accumulation once lactose not only can act as an inducer agent but also as a carbon source [22, 25]. When residual whey lactose was consumed, the volumetric rates of consumption of glycerol and lactose turned out to be quite different (Fig. 5b). Lactose consumption rate reached a maximum at 5 h and then slowly decreased, in coincidence with their exhaustion in the auto-induction culture medium (Fig. 5a). Glycerol consumption rates were not as high than those achieved with lactose but increased slowly until 9 h where it kept constant until the end of fermentation.

The use of auto-induction media containing glycerol and lactose under different aerobic conditions were investigated by several authors which observed that glycerol is preferentially consumed before lactose in an aerobic environment, which was not the situation observed in the shaken flasks used in our experiments. Higher aeration rates produce higher cell densities [18] and, consequently, a greater use of carbon and nitrogen sources [20, 22]. In our experiments, the employed shaken flasks presented low oxygen transfer rates which depend on different factors, such as culture medium volume, shape and size of the flask, agitation rate, bioprocess physicochemical conditions, among others. Therefore, if the biological oxygen demand is higher than the oxygen transfer capacity (through the vessel stopper and/or the gas–liquid interface), the system will be limited by O_2 and not by the carbon source producing an anaerobic environment [37].

Purification and Characterization of L-Arabinose Isomerase

Purification of recombinant *E. faecium* L-AI was performed by immobilized nickel affinity chromatography and the results obtained can be seen in Fig. 6.

Cell extracts showed a relevant increase in their specific activities after purification, indicating the presence of higher concentrations of target L-arabinose isomerase. Purified L-AI extracts obtained using IPTG, showed an enzyme specific activity of 0.33 U/mg, which turned out to be 3.3-fold higher than the specific activity of the crude extract (0.10 U/mg). Enzyme extracts obtained from auto-induction medium using commercial lactose, revealed an specific activity after purification of 1.5 U/mg, an improvement of 150% in comparison with the crude extract (0.6 U/mg).

The three times sonicated enzymatic extracts produced using residual whey lactose as inducer agent, showed the highest specific activity. Figure 6b displays the SDS–PAGE profile of the different samples collected during purification. By comparing lanes two (crude extract) and six (purified enzyme), it can be observed that there was a concentration of the protein of interest (around 55 kDa), as well as the removal of other contaminant proteins. Moreover, after purification, enzyme activity (2.4 U/mg) was about 4.5-fold higher when compared to the crude extract (0.53 U/mg), evidencing a significant improvement.

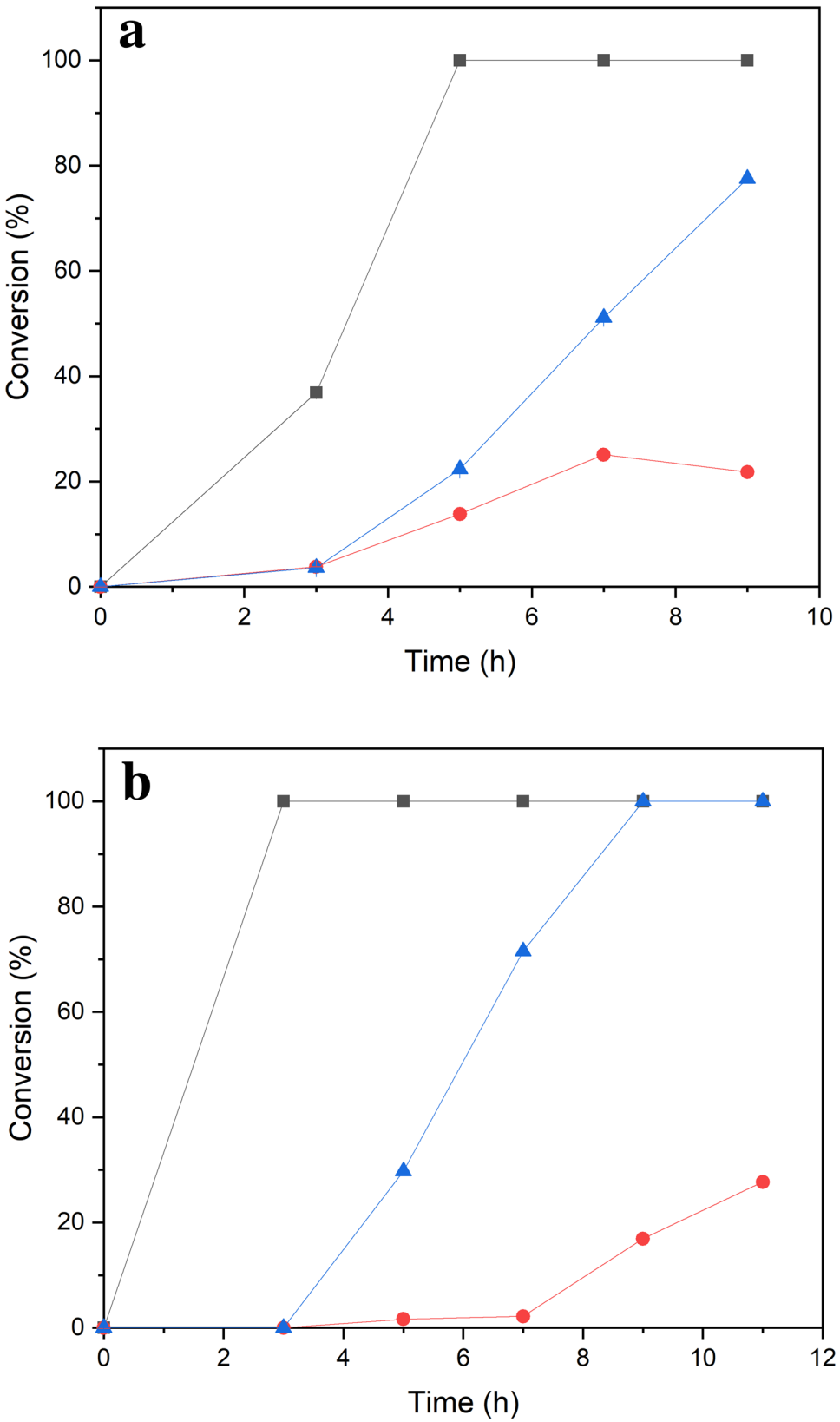

Fig. 4 Substrate uptake (conversion) during rL-AI production using auto-induction method with pure lactose: glucose (*filled black square*), glycerol (*filled red circle*), and lactose (2 g/L) (*filled blue triangle*). Commercial lactose (**a**) or residual whey lactose (**b**) were used as inducers (Color figure online)

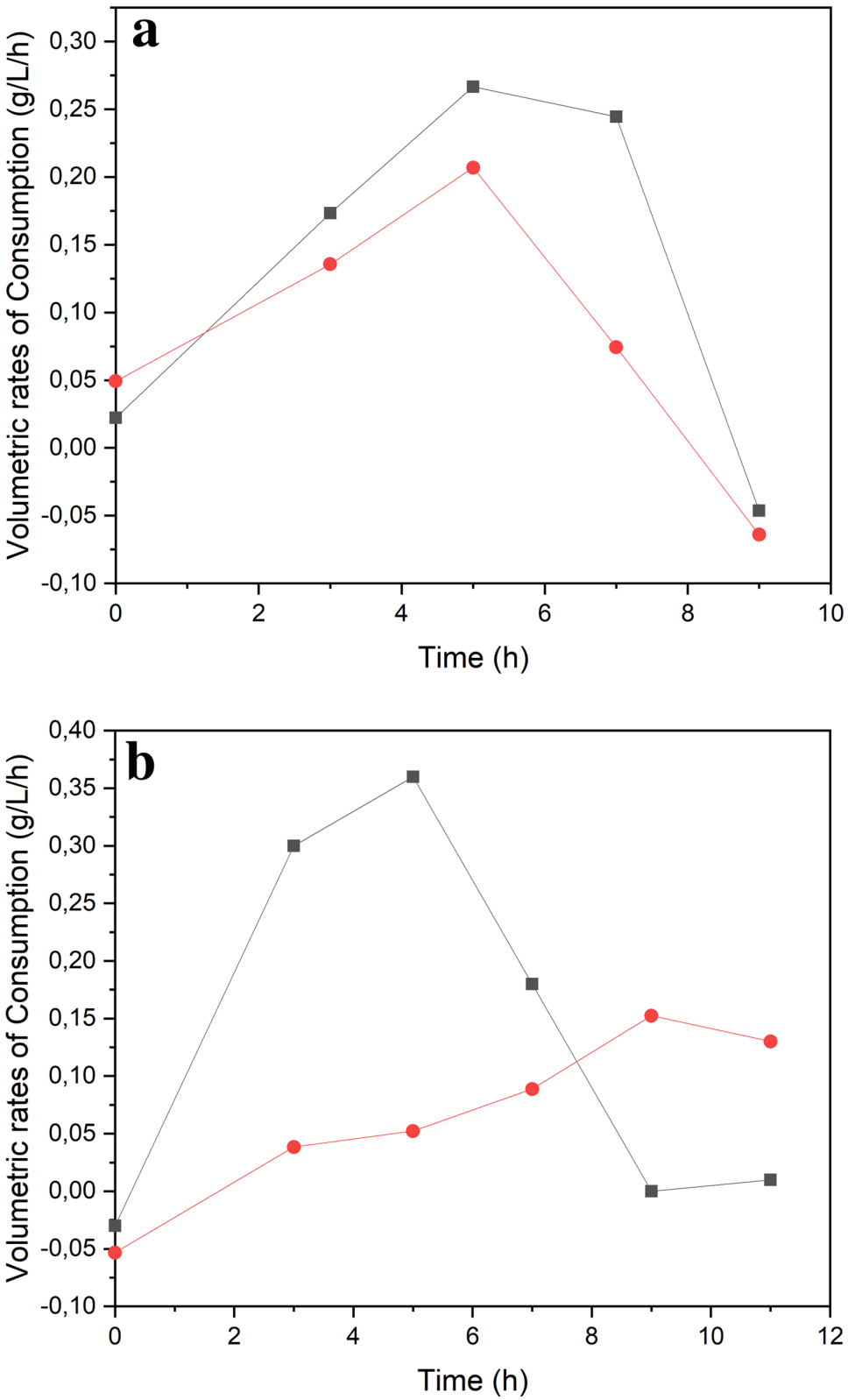

Fig. 5 Volumetric rates of consumption of substrates: lactose (*filled black square*) and (*filled red circle*) glycerol. **a** Commercial lactose and **b** residual whey lactose (Color figure online)

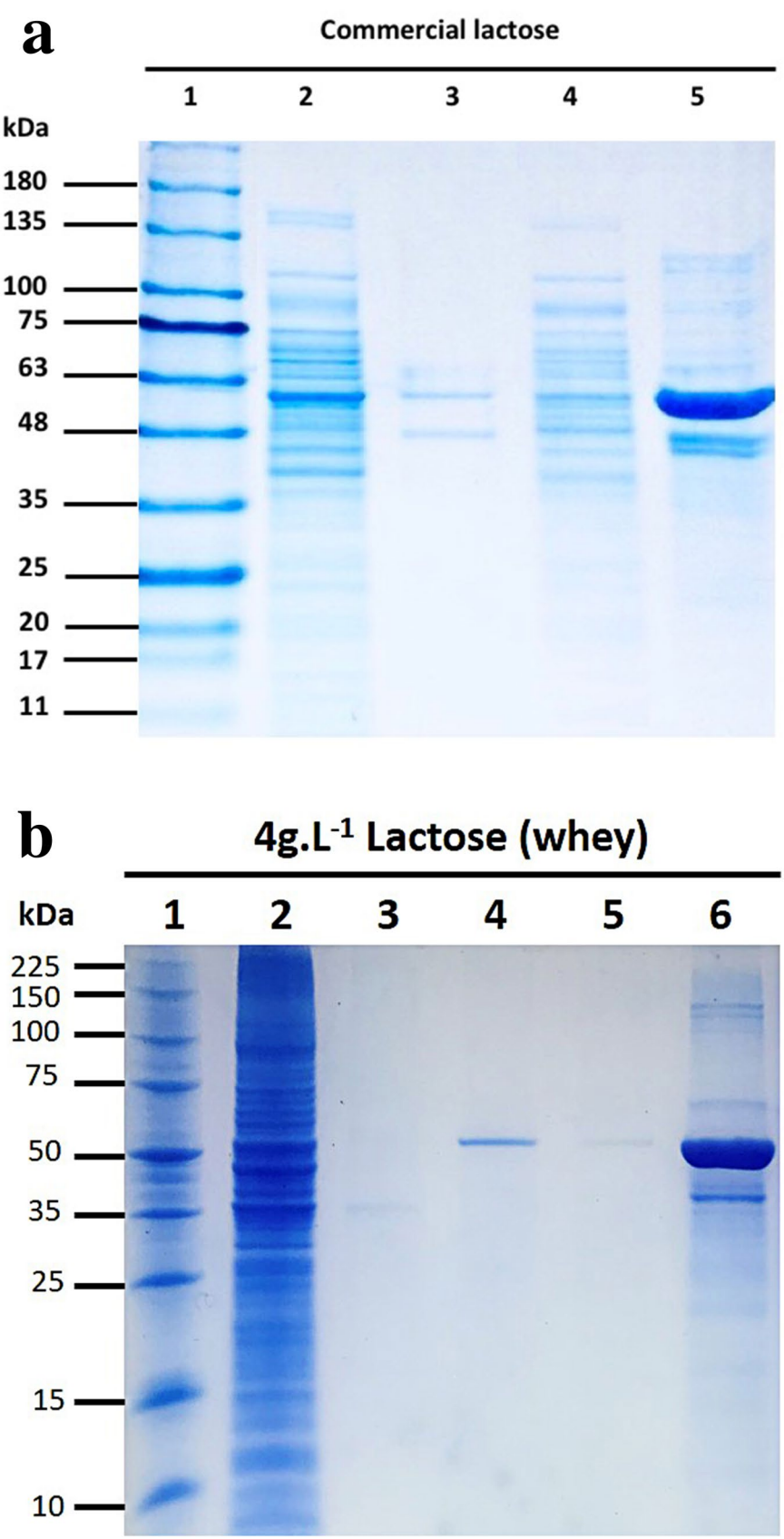

Fig. 6 SDS–PAGE profile of the protein fractions from L-arabinose isomerase extracts produced by auto-induction after each purification step. **a** Commercial lactose: *1* Molecular weight standards. *2* Fraction not retained in the Ni^{2+}-sepharose column (auto-induction). *3*, *4* Washings (auto-induction). *5* Purified enzyme (auto-induction). **b** Residual whey lactose: *1* Molecular weight standards. *2* Enzyme extract. *3* Fraction not retained in the Ni^{2+}-Sepharose column (whey). *4*, *5* Washings. *6* Purified enzyme

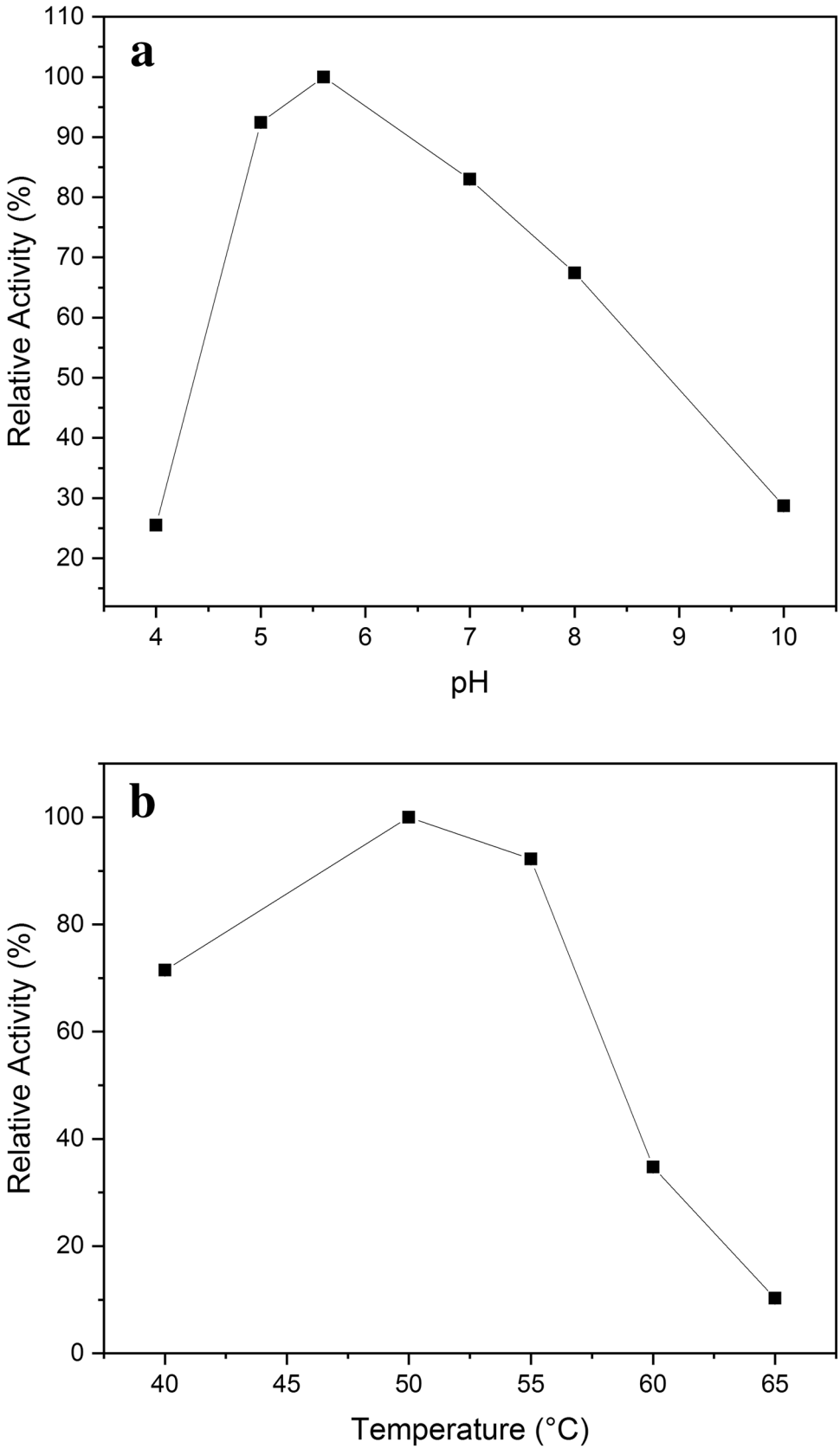

Fig. 7 **a** Effect of pH on enzymatic activity. Residual activity was measured under standard conditions (50 °C, 60 min). The activity at the optimal pH was defined as 100% (3.5 ± 0 U/mL). **b** Influence of temperature on catalytic activity. Activity measured at pH 5.6, after 60 min of reaction. The best enzymatic activity was defined as 100% (3.0 ± 0 U/mL)

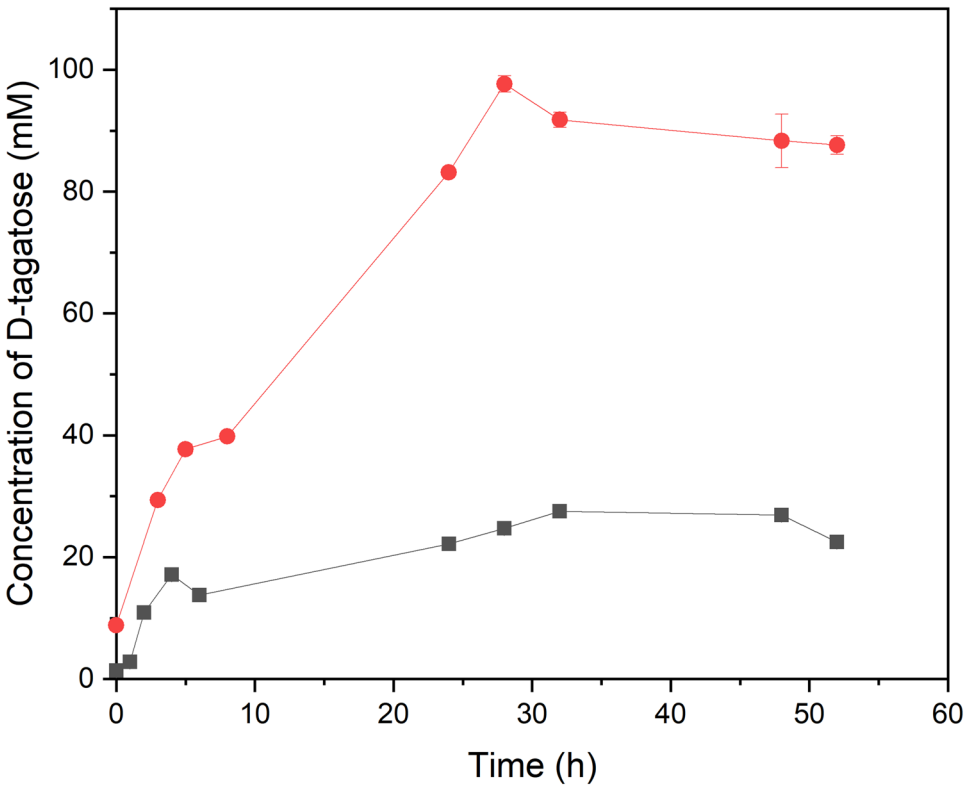

Fig. 8 Concentration of D-tagatose produced as a function of the reaction time using (*filled red circle*) 500 mM D-galactose and (*filled black square*) 164 mM D-galactose final concentrations (Color figure online)

Effect of pH on Catalytic Activity

The operational conditions of the isomerization reaction should be investigated, and, in these conditions, the enzyme should be able to allow high conversion yields, and fast reaction rates. Therefore, the pH dependence of rL-AI activity at a constant temperature of 50 °C was investigated and results can be observed in Fig. 7a.

The optimum pH was determined at an enzyme constant volume prepared in the presence of 1 mM $MnCl_2$. The enzyme retained more than 80% of its activity at pH values ranging from 5 to 7, with a maximum at pH 5.6.

Moderate to high temperature and acidic pH L-arabinose isomerases were reported to be appropriate for their use in D-tagatose industry production. Similarly to the results obtained in this work, other authors [12, 38] also reported profiles evidencing a decrease in enzyme activity as enzyme reaction pH was augmented. This interesting result reaffirms the use of enzymes with high activity at slightly acidic pHs for their employment at industrial level as suggested in the literature [11, 39]. Acidic pHs favor the reduction of undesirable effects to products such as browning and the formation of by-products, thereby generating a higher quality D-tagatose and a lower process cost overall. In that way, the use of lactose as inducer agent did not alter the already determined physicochemical behavior related to pH for rL-AI enzyme activity.

Effect of Temperature on Catalytic Activity

The stability of the purified rL-AI was evaluated at temperatures range of 40–65 °C for 60 min, Fig. 7b.

The optimum temperature was found to be 50 °C; however, the enzyme retained more than 90% of its activity at 55 °C. A rapid decline in their activity was observed for temperatures above 60 °C.

Regarding optimum temperature, previous reports from our group [12, 38] detected that of *E. faecium* DBFIQ E36 L-AI (expressed using IPTG) presented an optimum at 50 °C. The use of an auto-inducing medium, replacing IPTG by lactose, did not change optimum temperature for the catalytic activity.

Conversion of D-Galactose into D-Tagatose By L-Arabinose Isomerase

The production of D-tagatose from D-galactose was investigated at 55 °C and pH 5.6, using 0.114 U/mL (1.8 µM) of enzyme and two different D-galactose final concentrations (S_0): 164 and 500 mM, as shown in Fig. 8. Although rL-AI optimum temperature was 50 °C (Fig. 7b), temperatures of 55 °C were used in this assay, since more than 90% of total activity was retained.

The higher the reaction temperature was, the higher the obtained D-tagatose yield. Salonen et al. [40] and Xu

et al. [17] cited that the rate of D-tagatose production was increased as temperature also raised, because isomerization is an equilibrium reaction that is favored at higher temperatures. Due to the higher enzyme stability, a temperature of 55 °C was defined for the conversion tests, simulating conditions in which the enzyme can be applied industrially.

On the other hand, as seen in Fig. 8, it can be expected that higher concentrations of D-tagatose were achieved when higher D-galactose concentrations were assessed. In addition, D-tagatose concentration increased with time course reaction until the isomerization equilibrium was established. In this direction, when starting substrate concentration was of 164 mM, reaction equilibrium was established at 6 h, reaching a D-tagatose concentration of 13.8 mM and a D-galactose conversion of around 8.4%. However, when initial D-galactose concentration was of 500 mM, equilibrium was established at 28 h, reaching a production of 97.7 mM of D-tagatose and a D-galactose conversion around 20%.

Considering that the enzyme's source of origin employed in the present report was from a mesophilic microorganism, it can be said that the obtained conversion of D-galactose into D-tagatose was quite interesting, especially when compared with the results reported by other previously published L-arabinoses isomerases papers. For instance, Hung et al. [15] used a thermophilic L-AI from *Thermoanaerobacterium saccharolyticum* NTOU1 in the conversion D-galactose into D-tagatose, reaching a conversion of 20% after 10 h of reaction. Manzo et al. [12] achieved higher D-galactose conversions using the same enzyme, L-AI from *E. faecium*. However, the amount of enzyme used by these authors was approximately 8.3 times higher when compared the amount of L-AI used in this study. The amount of catalyst (enzyme) used in the reaction alters kinetics, but not its thermodynamic properties. In this case, when higher amounts of biocatalyst were used, equilibrium was attained before enzyme deactivation. Therefore, strategies to achieve higher conversions of D-galactose into D-tagatose should be further investigated. One possibility is to use immobilized enzymes, which allows not only the use of higher amounts of catalysts but also promotes their reusability. Preliminary results from our group indicates that L-AI cross-linked enzyme aggregates (CLEA) presented improved thermal and storage stabilities when compared to the soluble enzyme [41].

Conclusion

The goal of this study was to design an efficient and economical process to produce a recombinant L-arabinose isomerase from *E. faecium* DBFIQ E36 using *E. coli* BL21 (DE3) as host by submerged cultivation. Therefore, an induction medium previously proposed in the literature [22] was modified through the use of a cheap carbon source, such as lactose. Hence, commercial and residual whey lactose were successfully used for the production of this enzyme with expression levels higher to those obtained with IPTG. The complex medium proposed herein is a cheaper alternative and can be employed in simple auto-induction protocols. Likewise, the use of a relevant by-product from the dairy industry as an inducer agent has proved to be a viable and promising alternative that may allow a potential process scaling-up.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent All authors approved the manuscript and this submission.

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