Sans, C., García-Fruitós, E., Ferraz, R.M., González-Montalbán, N., Rinas, U., López-Santín, J., Villaverde, A., Álvaro, G.

Inclusion bodies of fuculose-1-phosphate aldolase as stable and reusable biocatalysts (2012) Biotechnology Progress, 28 (2), pp. 421-427.

Inclusion bodies of fuculose-1-phosphate aldolase as stable and reusable biocatalysts

Cristina Sans[1], Elena García-Fruitós[4,2], Rosa M Ferraz[2+], Núria González-Montalbán[2++], Ursula Rinas[5,6], Josep López-Santín[1*], Antonio Villaverde[2,3,4], Gregorio Álvaro[1]

Departament d'Enginyeria Química, Escola d'Enginyeria, Unitat de Biocatàlisi Aplicada associada al IQAC (CSIC), Universitat Autònoma de Barcelona, Edifici Q, 08193-Bellaterra, Spain.[1]

Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain[2]

Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, 08193, Bellaterra Barcelona, Spain[3]

CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), 08193, Bellaterra, Barcelona, Spain[4]

Helmholtz Centre for Infection Research, Inhoffenstr. 7, 38124 Braunschweig, Germany[5]

Leibniz University Hannover, Life Science - Technical Chemistry, Callinstr. 5, 30167 Hannover, Germany[6]

Running title: Aldolase inclusion bodies as biocatalysts

+ Present address: Bioingenium S.L.Edifici Hèlix, Parc Científic de Barcelona c/ Baldiri Reixac, 15-21. 08028-Barcelona, Spain

++ Present address: Center for Biomedical Engineering and Technology (BioMET). University of Maryland School of Medicine. 725 West Lombard Street, Room N370. Baltimore, MD 21201, USA

Corresponding author: Josep López-Santín. Departament d'Enginyeria Química. Escola d'Enginyeria. Universitat Autònoma de Barcelona, Edifici Q. 08193-Bellaterra (Cerdanyola del Vallès), Catalunya (Spain).

josep.lopez@uab.cat; Tel.:+34935811018; FAX:+34935812013

ABSTRACT

Fuculose-1-phosphate aldolase (FucA) has been produced in *E.coli* as active inclusion bodies (IBs) in batch cultures. The activity of insoluble FucA has been modulated by a proper selection of the producing strain, culture media and process conditions. In some cases, when an optimized defined medium was used, FucA IBs were more active (in terms of specific activity) than the soluble protein version obtained in the same process with a conventional defined medium, giving credit to the concept that solubility and conformational quality are independent protein parameters. FucA IBs have been tested as biocatalysts, either directly or immobilized into Lentikat® beads, in an aldolic reaction between DHAP and (S)-Cbz-alaninal, obtaining product yields ranging from 65 to 76 %. The production of an active aldolase as IBs, the possibility of tailoring IBs properties by both genetic and process approaches and the reusability of IBs by further entrapment in appropriate matrices fully support the principle of using self-assembled enzymatic clusters as tunable mechanically stable and functional biocatalysts.

INTRODUCTION

Aldolases are C-C bond forming enzymes with widespread applications. Stereoselective C-C bond catalysis by aldolases has been reported as an attractive alternative to conventional chiral organic chemistry methods for chemical and pharmaceutical industries. Aldolases catalyze C-C bond formation with defined stereochemistry yielding enantiomerically pure products, even when the starting materials are non-chiral substrates [1]. Fuculose-1-phosphate aldolase (FucA) is one of the four enzymes of the dihydroxyacetone phosphate (DHAP) dependent aldolase group, together with fructose-1,6-diphosphate, tagatose-1,6-diphosphate aldolase and rhamnulose-1-phosphate aldolase. These enzymes have been used as catalysts for the synthesis of enzyme inhibitors such as iminocyclitols, which have been largely investigated as therapeutic targets for the design of new antibiotics, antimetastic, antihyperglycemic or immunostimulatory agents [2-4]. Specifically, fuculose-1-phosphate aldolase catalyses the selective formation of R-R diols (D erythro compounds) by means of an aldol addition between DHAP and a wide variety of aldehydes. These aminopolyols are precursors of biologically active iminocyclitols [5,6]. FucA has been produced as a soluble recombinant fusion protein in *Escherichia coli* with a six-histidine tag under the control of isopropyl-D-thiogalactopyranoside (IPTG) inducible promoter *trc* [7].

Interestingly, some recent studies have demonstrated that, under certain circumstances, proteins can be produced as biologically active inclusion bodies (IBs) [8-13] observing that the conformational quality of proteins embedded in such aggregates is not significantly lower compared to their soluble counterparts. Moreover, these protein aggregates, can be easily recovered and purified from disrupted bacterial cells and employed, directly or in an immobilized form, as biocatalysts for bioprocess industry. [14-16]

In addition, it has been observed that the conformation quality of IBs produced in *E.coli* strains in absence of the main cytoplasmic chaperone DnaK is enhanced [17,18]. In fact, DnaK has not only an important role in the control of the distribution of functional protein species between soluble and insoluble cell fractions, but it also controls protein degradation, being the IBs formed larger and more biologically active than those formed in wild type strains [17].

The aim of this work is to demonstrate, as proof of concept, that enzymes like aldolases expressed as active IBs can be useful biocatalysts in synthesis reactions, since these aggregates are highly pure (most of IB biomass is expected to be the target protein), easy to produce and purify and even, they can be (re)used in immobilized form. At present, although some enzymes have been utilized in its aggregated form to catalyze reactions [16], it is still necessary to obtain more information about the conditions of active IBs production as well as the preparation and use of efficient IB-derived biocatalysts. In this sense, IBs can be employed directly or entrapped into particles (like Lentikats®, polyvinyl alcohol derived beads) in order to improve their mechanical and handling properties.

Therefore, we investigated here the conditions for the expression of FucA in active IBs and their characterization. On the other hand, the objectives include the preparation of IB-derived biocatalysts and their use in the stereoselective aldol addition between DHAP and (S)-Cbz-alaninal.

MATERIALS AND METHODS

Bacterial strains and vectors

The *fuc*A gene from *Escherichia coli* was expressed from a pTrcHis-based vector called pTrcfuc (Invitrogen, San Diego, USA) in *Escherichia coli* XL-1-Blue and JGT20 (Table

11). pTrcHis expression system is a commercial vector designed to express high levels of 2non-toxic proteins in *E.coli* under the control of *trc* expression promoter. The protein is 3produced with an N-terminal fused His6-tag.

4In M15ΔGlyA pQEαβT1fucA, the gene was expressed from pQEαβT1fucA (Fig.1), a 5pQE40-based plasmid (Qiagen) which was used to produce fuculose-1-phosphate 6aldolase in *E. coli* . It contains a *bla* gene, the *fucA* gene, a ColEI replication origin and 7a strong promoter T5. Furthermore, an rrnT1B terminator was introduced to ensure a 8better regulation (Sans et al., unpublished work).

9**Media and growth conditions**

10At small scale, bacterial cells were cultured in shake flask employing Luria-Bertani 11(LB) medium at different temperatures in order to control the deposition of FucA as 12IBs. The expression of *fuc*A gene was induced at 1-1.5 OD_{600} (0.3-0.45 g DCW/L) by 13the addition of IPTG to a final concentration of 1 mM. Samples were collected at 0 h, 3 14h, 5 h and 24 h after IPTG addition in order to follow the evolution of IBs formation. At 15bench scale, cells were grown in a 2-Liter bioreactor in an optimized defined medium 16(19,20) with a composition as follows, in g/L: Glucose (10.9), $(NH_4)_2HPO_4$ (4), 17KH_2PO_4 (13.3), Citric acid (1.55), $MgSO_4$ (0.59), Fe(III) citrate (0.1), $Na_2MoO_4.2H_2O$ 18(0.0021), CoCl2.6H2O (0.0025), $MnCl_2.4H_2O$ (0.015), $CuCl_2.2H_2O$ (0.0015), H_3BO_3 19(0.003), $Zn(CH_3COOH)_2.2H_2O$ (0.034), Titriplex III (0.014). The pH was adjusted with 20~ 2.7 g/L NaOH to pH 6.8.

21Bach cultures were carried out in a bioreactor Biostat B ® (Braun Biotech Int) equipped 22with a 2 L fermentation vessel. The pH was maintained at pH 7 ± 0.05 by adding 20% 23NH_4OH solution to the reactor. The temperature was kept at 37ºC throughout the 24cultivation. The pO_2 was maintained at 50% of air saturation by adapting the stirrer

speed between 450-1200 rpm and supplying air at 1.5 L/min. Induction was performed by a pulse of IPTG to a final concentration of 1 mM.

Inclusion bodies purification

The *E. coli* cells were harvested after cultivation by centrifugation for 15 min at 15,000 g, and the pellet stored at -80ºC. For sample processing, it was resuspended in lysis buffer (100 mM Tris- HCl, 150 mM KCl pH 7.5) at a ratio of 3 mL/ g dry cell weight (DCW). Besides, 15µL/g DCW of the proteases inhibitor phenylmethylsulfonyl fluoride (PMSF) were added from a 100 mM stock, followed by 80µL/ g DCW of lyzozyme from a 10 mg/ml stock, and the mixture incubated at 37ºC with shaking during 30-45 min. Later, nonylphenoxypolyethoxylethanol (NP-40) was added to a final concentration of 1% (v/v) and the mixture incubated at 4ºC for 30-45 min, followed by the addition of DNAse I (25 µL from a 1 mg/mL stock) and $MgSO_4$ (at a final concentration of 10 mM). After incubation with shaking at 37ºC for 30-45 min, the lysate was centrifuged at 15,000 g for 15 min and the supernatant was stored at -80ºC for further analysis of the soluble cell fraction.

The obtained pellet containing IBs, was washed by resuspension in 1 mL lysis buffer with Triton (2%) at 4ºC for 30-45 min followed by centrifugation for 15 min at 15000 g. The process was repeated several times until residual enzymatic activity was fully eliminated from the supernatant. The final pellet was stored at -80ºC.

Protein quantification

SDS-PAGE gels at 12% were prepared using MiniProtean II (Bio-Rad Laboratories Inc, California) equipment according to the manufacturer's instructions. Gels were stained with Coomassie Brilliant blue. The working conditions were 40 mA at 120 V for 90

1min. For quantification, Quantity One v.4.8 from Bio-Rad was used to determine the
2fraction of FucA in the sample.
3Protein concentration was determined with the Coomassie protein assay reagent kit
4(Pierce, Rockford, Ill, USA), using BSA as standard.
5
6**FucA enzymatic assay**
7Fuculose-1-phosphate aldolase activity was determined as previously reported [21] for
8both soluble and IBs enzyme. One unit of activity (AU) was defined as the amount of
9enzyme required to transform 1 μmol of fuculose-1-phosphate per minute at 25ºC under
10the assay conditions. The spectrophotometric measurement was performed with a
11Varian 300 spectrophotometer. For IBs activity determination, a stirred cuvette was
12employed to ensure sample homogeneity. Fuculose-1-phosphate was synthesized as
13dicyclohexylamine salt according to reported procedures [21].
14
15**Inclusion body immobilisation in Lentikat®**
16Entrapment of IBs in Lentikats ® was performed according to the protocol provided by
17Genialab (Lentikats® Tips and Tricks). Lentikat ® liquid, a polyvinyl alcohol (PVA)-
18based material, and Lentikat Stabiliser came from Genialab (Braunschweig, Germany;
19http://www.genialab.de/LentiDatasheets.php.)
20Briefly, the Lentikat® liquid was heated to 95ºC and then cooled to 40ºC. The enzyme
21preparation was added to Lentikat® liquid (proportion 1:4) and thoroughly mixed. The
22enzyme preparations consisted of an IBs suspension or pellet itself (after
23centrifugation). The resulting liquid was dropped by a Lentikat® Printer with 400 wires
24per 143 cm^2 (Genialab, Braunschweigh, Germany) into Petri dishes (diameter=14.5 cm),
25by dipping the tip of the wire into the solution and lifting it afterwards to get a uniform

shape and size of droplets. [22]. Lentikat® stabilizer solution was then poured into the gel particles for particle re-swelling and the whole mixture stirred for two hours. The lens-shaped particles were thoroughly washed with lysis buffer for two hours, filtered and weighted after removal of excess buffer, and either immediately used for bioconversion runs or stored at 4ºC until use.

FucA catalyzed aldol addition

The reaction between DHAP and (S)-Cbz-alaninal consisted of an aldol addition leading to the following product: (3R, 4R, 5S)-5-(benzyloxycarbonylamino-5,6-dideoxy-1-O-phosphonohex-2-ulose). Dihydroxyacetone phosphate dilithium salt ($DHAPLi_2$) was purchased from Fluka (Seelze, Germany).

(S)-Cbz-alaninal was synthesized in our laboratory by 2-iodobenzoic acid oxidation of the corresponding N-protected amino alcohol as reported in literature [21].

In order to ensure solubility of both substrates, dimethylformamide (DMF) was required as cosolvent. All aldol reactions were performed at 4ºC at initial concentrations of 25 mM DHAP and 42.5 mM (S)-Cbz-alaninal in 20% (v/v) DMF: 50 mM Tris-HCl, 150mM KCl pH=7 [21]. DHAP substrate was previously dissolved in aqueous buffer, whereas (S)-Cbz alaninal was dissolved in DMF. Reaction started after addition of the proper biocatalyst suspension to get the desired FucA activity (see Table 4) in a final reaction volume of 2.5 or 10 mL. The progress of the reaction was followed by measuring product, DHAP and (S)-Cbz-alaninal concentration evolution with time.

Dihydroxyacetone phosphate concentration was determined spectrophotochemically in an UV-VIS Cary (Varian) spectrophotometer [21].

(S)-Cbz-Alaninal and product concentration were measured in an Ultimate 3000 HPLC (Dionex) using an X-Bridge C18 5 μm column. Samples (20 μL) were withdrawn from

the reaction medium, dissolved with methanol (1:5) and analyzed subsequently by HPLC, using the following solvent system: solvent A: 0.1% v/v trifluoroacetic acid (TFA) in H_2O, solvent B: 0.095% v/TFA in H2O:CH_3CN 1:4 v/v; gradient elution from 10 % to 70 % B in 30 min, flow rate 1 cm^3/min, UV detection at 215 nm.

RESULTS AND DISCUSSION

Two different expression systems were available in our laboratory for soluble FucA production (see Materials and Methods for details). In the first one, FucA was expressed in *E.coli* XL1 Blue driven by the plasmid pTrcFucA. No IBs were detected during growth at 37ºC, after induction of gene expression by 100 μM IPTG [23]. In the second one, the *E. coli* K-12 derived strain M15 ΔglyA, harboring a low copy number plasmid from pQE-40 (Qiagen), was used for soluble FucA overexpression. This is a glycine auxotrophy-based system to ensure plasmid stability which avoids the need of antibiotic supplementation [24]. Transcription of the gene under the control of the strong T5 promoter resulted in higher soluble aldolase activities than the first strain, working at the same conditions, without significant IBs formation (unpublished results).

Formation of active FucA inclusion bodies from XL1 Blue and JGT20

In order to investigate the feasibility of FucA expression as active IBs, as a first approach, the plasmid pTrcfucA was transformed in *E. coli* JGT20, a $DnaK^-$ mutant (see Materials and Methods). $DnaK^-$ background minimizes proteolysis of aggregation-prone recombinant proteins and stimulates their deposition as functional protein species in IBs[25, 17, 26].

For comparison purposes, transformed XL-1-Blue and JGT20 strains were cultured in shake flasks in rich medium (LB), at two temperatures, 37 and 42ºC. Gene expression

was induced by an IPTG pulse at higher concentration (1 mM), and samples were collected at different times after induction.

IBs with aldolase activity were observed in all cases (Table 2), being the amount of active enzyme per gram of dry cell weight (DCW) in the *dnaK* mutant much higher than in *E. coli* XL1 Blue, at both temperatures.

In XL1 Blue, the levels of soluble FucA were, according to previous work, around 280 AU/g DCW [7], and only a reduced fraction of FucA (around 2 %) was detected in the IBs at both temperatures. In the case of JGT20, higher protein amounts were observed in soluble and in insoluble forms. Soluble levels reached more than 2000 AU/g DCW, and the IBs fraction represented around 4% of the total recombinant protein at 37ºC, and only 2% at 42º C.

In XL1 Blue, the specific activity (AU/mg protein) of IBs was higher at 42ºC than at 37ºC with a maximum value of around 3 AU/mg (data not shown). The purity of the IBs was relatively low in both cases, as only 25-30% of the total IBs protein corresponded to full-length FucA (data not shown).

Concerning the *dnaK* background, data are shown in Figure 2 for 37ºC growth (final biomass concentration 1.6 g/L) and 42ºC (final biomass concentration 0.6 g/L). The JGT20 strain rendered higher values of FucA specific activity than the XL1 Blue strain at 37ºC, as 6 AU/mg of target protein were found in IBs. These results are in accordance to the suggestion that active IBs can have equal or higher specific activity than the soluble fraction[11,14] as it has been reported that misfolds may be more abundant sometimes in the soluble than the more dense IB fraction[11].

It has to be noticed that the increase of temperature to 42ºC had a negative effect on the activity of IBs FucA in JGT20. In fact, it has been reported that at lower temperatures,

metabolism is slower leading to slower translation and folding processes. Slower aggregation of proteins could lead to higher yields of correctly folded proteins [13].

The IBs in JGT20 were of higher purity than in XL-1-Blue, as 70% of protein in IBs was recombinant aldolase at both temperatures. Consequently, the results demonstrate that JGT20 is able to render active FucA IBs. Nevertheless, although specific activity and purity at 37ºC were good enough, the feasibility of using JGT20 strain in the presented conditions for FucA expression in IBs was very compromised by the low yield (only 4% of total enzyme in IB).

A possible improvement of IBs yield could be eventually reached by changing the expression strain and/or employing different growth conditions [27, 28]. The use of an optimized defined medium (see Materials and Methods) did not improve the yield of active inclusion bodies in JGT20 strain. Consequently, different expression strains were tested.

Inclusion body production with M15ΔGlyApQEαβT1fucA strain.

The auxotrophy-based system (strain M15ΔGlyApQEαβT1fucA) was also evaluated for FucA production in form of IBs. This strain had been employed for soluble RhuA and FucA production in high-cell density cultures, giving around 1000 AU/g DCW [24] (unpublished results). The most relevant features of this strain are given in the Materials and Methods section.

Growth of the above strain and FucA IBs formation were studied in shake flasks experiments. In LB medium, the expression was again mainly in soluble form as for JGT20 strain. Nevertheless, employing the optimized defined medium, expression was mainly directed to IBs. In this case, active inclusion bodies represented 57 % of the total aldolase produced.

Then, 2L-batch cultivations were performed to characterize the IBs obtained using the optimized defined medium and to produce the necessary amounts for their evaluation as biocatalysts. Cells were grown at 37ºC and FucA production induced by an IPTG pulse of 1 mM when the culture reached 3 units of optical density (OD_{600}). The culture broth was centrifuged and cells harvested (3 g/L final biomass concentration). IBs and soluble intracellular FucA were obtained using lysozyme treatment. An SDS-PAGE showing total, soluble and insoluble fractions is presented in Figure 3. A pre-induction sample was also included as expression control.

As can be seen in Figure 3, FucA is mainly produced as IBs. 450 AU/g DCW were found in form of IBs in front of 339 AU/g DCW in the soluble fraction. The specific activity of IB was 8.5 AU/mg FucA. On the other hand, IBs showed a moderate purity of 34% of the total protein, due to the expression vector employed [24]. The obtained IBs suspension was used in further immobilization trials and also as biocatalyst in aldol addition reactions.

Immobilized inclusion bodies as biocatalysts

Active IBs can be employed directly as "self-immobilized" biocatalysts in bioconversions [14]. Nevertheless, in order to solve drawbacks like the difficulty in handling, they can also be employed entrapped in beads [29]. Both approaches have been employed in this work.

Encapsulation in Lentikat® particles has been chosen for the preparation of entrapped IBs. A previous crosslinking by glutaraldehyde was assayed in order to improve IBs handling properties, but the addition of this reagent resulted in a drastic loss of activity (data not shown). Consequently, IBs were directly encapsulated in Lentikat beads.

Results from a IBs Lentikats immobilization experiment are shown in Table 3. IBs containing 51 AU were immobilized (see Materials and Methods), and 2.9 g of Lentikat® particles were obtained. The activity test of the immobilized derivatives gave 40.53 AU/g of beads, what means an apparent total activity in the lentils of only 1.54 AU (3 % of the original enzymatic load). After two hours of incubation, there was no significant leakage of aldolase activity from the beads (see Table 3), showing that IBs size was high enough to avoid exit from the particles. Therefore, the low activity measured in the beads has to be attributed either to a possible enzyme deactivation in the process (not expected at the operating conditions) or, more probably, to an apparent reduction in activity, due to diffusional limitations.

Use of IBs biocatalysts in synthesis

The aldol addition between DHAP and (S)-Cbz-alaninal catalyzed either by direct IBs, Lentikat®-immobilized IBs or soluble FucA, was studied under comparative conditions (the same nominal or assay activity measured with the FucA standard assay). Table 4 shows the results for the initial rate of DHAP disappearance and the reaction yields measured as DHAP conversion degree and product yield after 25 h.

As it can be seen, soluble fraction and IBs exhibit the same initial rate. This is an indication of non diffusional limitations using IBs directly. On the other hand, the Lentikat®-immobilized IBs presented higher initial rate than the corresponding to the nominal activity. This means that most of the offered enzyme activity was really actively immobilized and that diffusional limitations have less influence in the synthesis reaction with non natural substrates than with the natural one employed in the standard assay [30]. Reaction rate is much faster in the assay reaction than in the aldol condensation

1one. Nevertheless, diffusion is still the rate-controlling step as the reaction rate is only 5
2times higher than obtained with the soluble enzyme (18/3.5 = 5.14) and the activity of
3the total immobilized enzyme would correspond to 33 times higher (obtained by
4dividing 51/1.54). A similar behavior was observed with a further reaction performed
5with less charged Lentikat® particles (0.2 AU/mL), as is shown in Table 4.
6The concentration profiles for the Lentikat-IBs catalyzed reaction at 1.5 AU/mL
7nominal activity are presented in Figure 4. Lentikat-entrapped IBs have shown to be
8good catalysts for the target aldol addition, because of the high conversions obtained.
9Furthermore, they are very stable biocatalysts as they still showed full activity two
10months after preparation indicating negligible enzyme inactivation and leakage from the
11beads.
12
13**CONCLUSIONS**
14It has been demonstrated that Fuculose-1-phosphate aldolase can be expressed as active
15IBs. Employing a DnaK$^-$ mutant strain, the obtained IBs had a high purity, but most of
16the protein was expressed in soluble form. Re-direction of expression towards active
17IBs has been possible by employing appropriate strain and culture medium, improving
18yield and specific activity.
19FucA IBs have shown to be appropriate biocatalysts for aldol addition reactions, with
20similar characteristics than the soluble enzyme. The biocatalyst mechanical properties
21have been improved by entrapment into Lentikat particles. Although the reaction
22between DHAP and (S)-Cbz-alaninal is diffusionally limited, product yields, reaction
23rates and stability are good enough for its practical implementation.

To sum up, IBs are an interesting alternative to use fuculose-1-phosphate aldolase as biocatalyst because they contain the enzyme in high activity and purity and they could potentially be (re)used efficiently in synthetic reactions.

ACKNOWLEDGMENTS

This work has been supported by the Spanish MICINN, projects CTQ2008-00578 and BFU2010-17450, and by DURSI (2009SGR281 and 2009SGR108), Generalitat de Catalunya. The Department of Chemical Engineering of UAB constitutes the Biochemical Engineering Unit of the Reference Network in Biotechnology of the Generalitat de Catalunya (XRB). We also appreciate the support from CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN, Spain), an initiative funded by the VI National R&D&i Plan *2008-2011*, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund.

C.S. acknowledges UAB for a predoctoral grant as well as DAAD for a fellowship which made possible to collaborate with the Institute of Technical Chemistry (Hannover, Germany). AV has been distinguished with an ICREA ACADEMIA award.

REFERENCES

1. Samland AK, Sprenger GA. Microbial aldolases as C-C bonding enzymes-unknown treasures and new developments. *Appl Microbiol Biot*. 2006; 71: 253-264.
2. Clapés P, Fessner WD, Sprenger GA, Samland AK. Progress in stereoselective synthesis with aldolases. *Curr Opin Chem Biol*. 2010; 14: 154-157.
3. Fechter MH, Stutz AE, Tauss A. Chemical and chemo-enzymatic approaches to unnatural ketoses and glycosidase inhibitors with basic nitrogen in the sugar ring. *Curr Org Chem.* 1999; 3: 269-285.

14. Koeller KM, Wong CH. Complex carbohydrate synthesis tools for glycobiologists: 2Enzyme-based approach and programmable one-pot strategies. *Glycobiology*. 2000; 10: 31157-1169.
45. Espelt L, Parella T, Bujons J, Solans C, Joglar J, Delgado A, Clapes, P. 5Stereoselective aldol additions catalyzed by dihydroxyacetone phosphate dependent 6aldolases in emulsion systems: Preparation and structural characterization of linear and 7cyclic aminopolyols from aminoaldehydes. Chem-*Eur J* 2003; 9: 4887-4899.
86. Espelt L, Bujons J, Parella T, Calveras J, Joglar J, Delgado A, Clapes P. Aldol 9additions of dihydroxyacetone phosphate to N-Cbz-amino aldehydes catalyzed by L-10fuculose-1-phosphate aldolase in emulsion systems: Inversion of stereoselectivity as a 11function of the acceptor aldehyde. *Chem Eur J*. 2005; 11: 1392-1401.
127. Durany O, Caminal G, de Mas C, López-Santín J. Studies on the expression of 13recombinant fuculose-1-phosphate aldolase in *E. coli*. *Process Biochem.* 2004; 39: 141677-1684.
158. Jevsevar S, Gaberc-Porekar V, Fonda I, Podobnik B, Grdadolnik J, Menart V. 16Production of nonclassical inclusion bodies from which correctly folded protein can be 17extracted. *Biotechnol Prog.* 2005; 21: 632-639.
189. García-Fruitós E, Carrió MM, Arís A, Villaverde A. Folding of a misfolding-prone 19β-galactosidase in absence of DnaK. *Biotechnol Bioeng.* 2005; 90: 869-875.
2010. Ventura S, Villaverde A. Protein quality in bacterial inclusion bodies. *Trends* 21*Biotechnol.* 2006; 24: 179-185.
2211. González-Montalban N, García-Fruitós E, Villaverde A. Recombinant protein 23solubility- does more mean better? *Nature Biotechnol.* 2007; 25: 718-720.
2412. Peternel, S; Grdadolnik, J; Gaberc-Porekar, V, et al. Engineering inclusion bodies 25for non denaturing extraction of functional proteins *Microbial Cell Factories* 2008; 7: 2634.
2713. Peternel S, Gaberc-Porekar V, Komel R. Bacterial growth conditions affect quality 28of GFP expressed inside inclusion bodies. *Acta Chim Slov.* 2009; 56: 860-867.

114. García-Fruitós E, González-Montalbán N, Morell M, Vera A, Ferraz RM, Arís A,
2Ventura S, Villaverde A. Aggregation as bacterial inclusion bodies does not imply
3inactivation of enzymes and fluorescent proteins. *Microb Cell Fact.* 2005; 4: 27.
415. Nahálka J, Gemeiner P. Thermoswitched immobilization-A novel approach in
5reversible immobilization. *J Biotechnol.* 2006; 127: 478-482.
616. Nahálka J, Vikartovská, A, Hrabárová E. A crosslinked inclusion body process for
7sialic acid synthesis. *J Biotechnol.* 2008; 134: 146-153.
817. García-Fruitós E, Aris A, Villaverde A. Localization of functional polypeptides in
9bacterial inclusion bodies. *Appl Environ Microbiol*. 2007; 73: 289-294.
1018. González-Montalbán N, García-Fruitós E, Ventura S, Arís A, Villaverde A. The
11chaperone DnaK controls the fractioning of functional protein between soluble and
12insoluble cell fractions in inclusion body-forming cells. *Microb Cell Fact*. 2006; 5:26.
1319. Korz DJ, Rinas U, Hellmuth K, Sanders EA, Deckwer WD. Simple fed-batch
14technique for high cell density cultivation of *Escherichia coli*. *J Biotechnol.* 1995; 39:
1559–65.
1620. Li Z, Kessler W, van den Heuvel J, Rinas U. Simple defined autoinduction medium
17for high level recombinant protein production using T7-based *E. coli* expression
18systems *Appl Microbiol Biotechnol.* 2011; accepted.
1921. Suau T, Álvaro G, Benaiges MD, López-Santín J. Influence of secondary reactions
20on the synthetic efficiency of DHAP-aldolases. *Biotechnol Bioeng.* 2006; 93: 48-55.
2122. Durieux A, Nicolay X, Simon JP. Continuous malolactic fermentation by
22*Oenococcus oeni* entrapped in Lentikats. *Biotechnol Lett.* 2000; 22:1679-1684.
2323. Durany O, De Mas C, López-Santín J. Fed-batch production of recombinant
24fuculose-1-phosphate aldolase in *E.coli. Process Biochem*. 2005; 40: 707-716.
2524. Vidal L, Pinsach J, Striedner G, Caminal G, Ferrer P. Development of an antibiotic-
26free plasmid selection system based on glycine auxotrophy for recombinant protein
27overproduction in *Escherichia coli. J. Biotechnol.* 2008, 134:127–136.
2825. Carrió MM, Villaverde A. Localization of chaperones DnaK and GroEL in bacterial
29inclusion bodies. *J Bacteriol.* 2005; 187: 3599-3601

126. González-Montalban N, Natalello A, García-Fruitòs E, Villaverde A, Doglia SM. *In*
2*situ* protein folding and activation in bacterial inclusion bodies. *Biotechnol Bioeng.*
32008; 100: 797-802.
427. Fahnert B, Lilie H, Neubauer P. Inclusion Bodies: Formation and utilisation. *Adv*
5*Biochem Eng Biot*. 2004; 89: 93-142.
628. Kopetzki E, Schumacher G, Buckel P. Control of formation of active soluble or
7inactive α-glucosidase in *E.coli* by induction and growth conditions. *Mol Gen Genet.*
81989; 216: 149-155.
929. Nahálka J, Nidetzky B. Fusion to a pull-down domain: a novel approach of
10producing Trigonopsis variabilitis D-aminoacid oxidase as insoluble enzyme
11aggregates. *Biotechnol Bioeng*. 2007; 97: 454-461.
1230. Suau T, Álvaro G, Benaiges MD, López-Santín J. Performance of an immobilized
13fuculose-1-phosphate aldolase for stereoselective synthesis. *Biocatal Biotransformation*
142009; 27: 136-142.

FIGURE CAPTIONS

Fig. 1. pQEαβfucAT1 vector diagram.

Fig. 2. Specific activity (AU/mg FucA) and purity (%FucA) in IBs and soluble fraction (SF) for JGT20 strain at 37ºC (a) and 42ºC (b). Error bars correspond to the standard error of 3 replica.

Fig.3. SDS-PAGE of protein expression from batch culture of M15ΔGlyApQEαβT1fucA on optimized defined medium at 37ºC: preinduction sample (lane 1), insoluble fraction (lane 2), soluble fraction (lane 3), total fraction (lane 4) and molecular weight markers (lane M).

Fig.4. Time course of aldol condensation reaction catalyzed by Lentikat-IBs beads. Reaction was performed in DMF/buffer (1:4 v/v) at an initial aldolase activity of 1.5 AU/mL reactor (see Materials and Methods for reaction medium composition).