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A Mild Procedure for Enone Preparation Catalysed by Bovine Serum Albumin in a Green and Easily Available Medium

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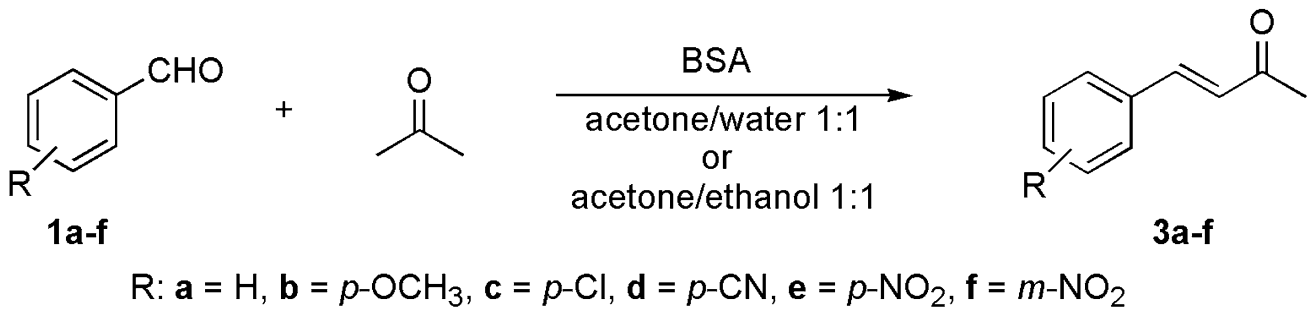
Abstract
A simple and mild procedure to obtain α,β-unsaturated ketones from acetone and a set of benzaldehydes is described. The approach applies bovine serum albumin (BSA) catalysis and water or ethanol, this mild reaction medium contrasting with the strong reaction conditions of the classic aldol condensation. Except for the assayed nitrobenzaldehydes, high enone yields (88–97%) were attained. In addition to its mildness, further advantages of this procedure are the use of a green catalyst exhibiting an efficient reuse and the use of eco-friendly and cheap solvents. In order to gain a deeper understanding of the involved catalytic mechanism, computational experiments on BSA structural analysis and molecular docking were carried out.

Graphical Abstract



Keywords Bovine serum albumin · Catalytic promiscuity · Cross aldol condensation · Enones

1 Introduction

The cross aldol condensation, also known as Claisen–Schmidt reaction, is a carbon–carbon bond-forming reaction conducting to α,β-unsaturated carbonyl compounds (**3a–f**), which are formed by dehydration of the intermediate aldol addition product **2a–f** (Scheme 1). Although it is a well-known transformation, it still receives attention because of the synthetic versatility of the α,β-unsaturated carbonyl moiety; for instance, aromatic enones are precursors of diverse pharmacologically active products [1, 2]. However, conventional aldol condensation is usually carried out in a strong and hot basic medium, which affords subproducts such as bis α,β-unsaturated carbonyl compounds and Michael addition aducts [3, 4]. To circumvent this problem, reported alternatives include

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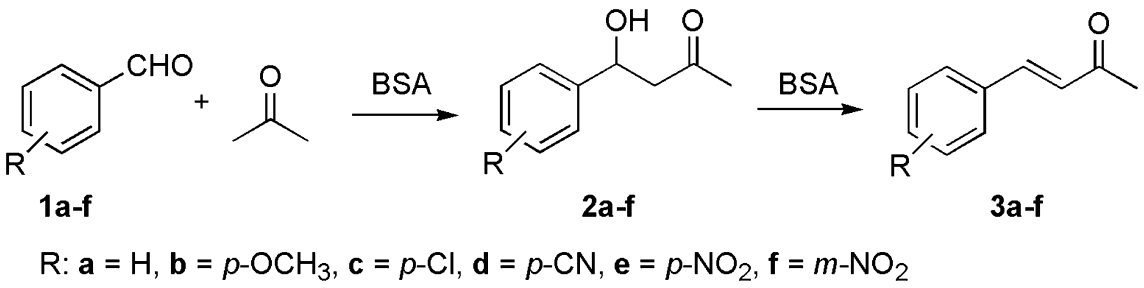

Scheme 1 BSA-catalysed aldolic reaction of benzaldehydes **1a–f** with acetone

microwave irradiation or metal ion catalysis, but many of them involve hazardous or expensive reagents and solvents [5].

Biocatalytic procedures are currently integrated into synthetic chemistry because they provide environmentally friendly and sustainable transformations, carried out under mild reaction conditions. Focusing on aldol reactions, aldolases usually display high stereoselectivity but limited aldol donor acceptance [6]. Catalytic promiscuity, the ability of an enzyme to catalyse reactions mechanistically different to those involved in vivo [7, 8], has expanded the synthetic opportunities of biocatalysts. Several hydrolases, mainly commercially available lipases and proteases, have exhibited promiscuous activity and variable enantioselectivities in the cross aldol addition [9–12]; recently, an enzyme isolated from *Daucus carota* root has also been reported to catalyse enantioselectively the cross aldol addition of acetone to a set of benzaldehydes [13]. In relation to the cross aldol condensation, the enzyme 4-oxalocrotonate tautomerase (4-OT) has displayed promiscuous activity in the aldol addition of benzaldehyde to acetaldehyde and its subsequent dehydration to cinnamaldehyde; further engineering of 4-OT has conducted to cinnamaldehyde in 50% yield [14]. Additionally, preparation of enones using a D-aminoacylase and imidazole as co-catalyst has also been reported [15].

In addition to enzymes, non-enzymatic proteins such as albumin are also able to promote organic reactions even if they do not possess a true catalytic site. In particular, commercially available bovine serum albumin (BSA) has been applied to catalyse different reactions such as the Morita–Baylis–Hillman reaction [16] and other carbon–carbon bond-forming reactions, ketone reduction, sulfide oxidation and it has been defined as a promiscuous biocatalyst [17]. In regard to the aldol addition step in cross aldol reactions, BSA exhibited aldolase activity with moderate enantioselectivity in the addition of acetone to 6-methoxy-2-naphtaldehyde in neutral aqueous solution; benzaldehyde and other substitued benzaldehydes were less efficiently recognised by the biocatalyst [18]. On the other hand, in the ionic liquid 1-butyl-3-methyl imidazolium bromide, BSA promoted the condensation of acetone with a set of substitued benzaldehydes, giving the corresponding α,β-unsaturated carbonylic compounds in good and high yields [19].

Taking into account the commented interest on mild, green and inexpensive procedures to get α,β-unsaturated carbonyl compounds and considering the above outlined antecedents, we studied the BSA-catalysed cross aldol condensation of a set of benzaldehydes (**1a–f**, Scheme 1) with acetone in non-expensive solvents and in this work we report the obtained results.

2 Materials and Methods

2.1 General

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (fraction V) and used straight from the bottle except for control experiments (Table 1). All reagents and solvents were of analytical grade and purchased from commercial suppliers. Benzaldehyde was purified by filtration through basic aluminium oxide, followed by distillation. Absolute ethanol was employed and prior to use, acetone was dried over potassium carbonate and distilled.

Table 1 Control experiments for BSA-catalysed benzalacetone (**3a**) formation

Entry	Biocatalyst	Conversion[a]
1	–	0
2	BSA	54
3	BSA pretreated with urea at 100 °C[b]	1

Reactions were carried out in acetone/water 1:1 at 30 °C and a BSA/**1a** (mass ratio) = 5.2 (See Sect. 2)

[a]Determined by HPLC at 120 h

[b]Prior to the assay, BSA (100 mg) was stirred in 8 M aqueous urea (2 ml) for 24 h at 100 °C, then evaporated under reduced pressure. To remove urea, the resulting residue was washed with acetone/water 1:1 (3 × 5 ml) and dried

Reactions were monitored by thin-layer chromatography with Silicagel 60 F_{254} plates (Merck); plates were developed using hexane/ethyl acetate 70:30 v/v and visualised with UV light. Flash column chromatography was performed employing 0.040–0.063 mm Silicagel 60 (Merck).

HPLC analyses were carried out using a C18 column (length: 150 mm; internal diameter: 4.6 mm; particle size: 5 µm). Analysis of aliquots from biotransformations of **1a–c,e,f** was done applying a 4 min methanol/water gradient from 60:40 v/v (2 min) to 75:25 v/v (1 min) at a flow rate of 0.9 ml min^{-1} and detection at 274 nm. Retention times were: **1a**: 3.9 min; **3a**: 5.6 min; **1b**: 4.6 min; **3b**: 6.3 min; **1c**: 7.1 min; **3c**: 9.1 min; **1e**: 3.8 min; **2e**: 2.9 min; **3e**: 4.9 min; **1f**: 3.6 min; **2f**: 3.0 min; **3f**: 5.1 min: Samples involving **1d** were analysed by a 3 min gradient of a mixture of the same solvents from 60:40 v/v (2 min) to 70:30 v/v (1 min) at the same flow rate and detection at 254 nm. Retention times were: **1d**: 2.9 min; **2d**: 2.4 min; **3d**: 3.7 min.

Aldols **2d–f** were analysed by GC using a chiral column CP-Chirasil-Dex (25 m × 0.32 mm × 0.25 µm). Oven temperature was set at 100 °C (1 min), increased to 185 °C (5 min) and maintained at the final temperature for 25 min.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance II spectrometer at 500 and 125 MHz, respectively; chemical shifts (δ) are reported in ppm from TMS and $CDCl_3$ as solvent. The following abbreviations were used to indicate the multiplicity: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad signal.

2.2 BSA-Catalyzed Aldol Condensation of Compounds 1a–f

2.2.1 General Procedure for BSA-Catalysed Aldol Condensation in Analytical Scale

Experiments reported in Tables 1, 2, 3 and 4 involved a solution of the aldehyde (**1a–f**, 0.05 mmol; for instance, 5 µl of **1a**), the indicated solvent or mixture of solvents (1 ml) and BSA at the indicated BSA/**1a–f** mass ratio (for instance, 54 mg for BSA/**1a** = 10.4). The resulting mixtures were shaken at 200 rpm and at the indicated temperature; aliquots from the biotransformations were withdrawn at different times and after centrifugation, analysed by TLC and HPLC.

2.2.2 BSA-Catalysed Preparation of Products 2d–f and 3a–f

Following the above reported analytical procedure, BSA-catalysed preparative biotransformations were conducted by adding the biocatalyst (540 mg) to a solution of the corresponding aldehyde **1a–f** (0.5 mmol) in acetone/water 1:1 v/v (10 ml). To obtain product **2d** from **1d**, acetone/ethanol 1:1 v/v was employed instead of acetone/water 1:1 v/v. The mixtures were shaken at 45 °C and 200 rpm for the below reported time and then, BSA was filtered off and washed with acetone (5 ml). The filtrates were concentrated to remove acetone under reduced pressure and extracted with ethyl acetate (4 × 5 ml), the organic phases were dried over sodium carbonate, filtered and the solvent eliminated in vacuo. Silicagel flash column chromatography of the resulting residue, using the below indicated elution solvents, afforded products **2d–f** and **3a–f**.

Table 2 Cosolvent content effect in BSA-catalysed benzalacetone (**3a**) formation

Entry	Acetone:cosolvent ratio	Conversion[a] (%)
1	Acetone	21
2	Acetone/ethanol 90:10	33
3	Acetone/ethanol 85:15	37
4	Acetone/ethanol 75:25	40
5	Acetone/ethanol 50:50	51
6	Acetone/ethanol 25: 75	31
7	Acetone/ethanol 15:85	34
8	Acetone/ethanol 5:95	39
9	Acetone/water 95:5	41
10	Acetone/water 90:10	44
11	Acetone/water 80:20	56
12	Acetone/water 75:25	59
13	Acetone/water 50:50	54
14	Acetone/water 25:75	10

Reactions were carried out at 30 °C and at a (BSA/**1a**) mass ratio = 5.2. (See Sect. 2)

[a]Determined by HPLC at 120 h

2.2.2.1 ***4-(1-Hydroxy-3-oxobutyl)benzonitrile*** **(2d)** Reaction time: 72 h; elution solvent hexane/ethyl acetate 80:20 v/v, yield: 54%; R_f = 0.39 (hexane/ethyl acetate 70:30). ^{1}H NMR: δ 7.63 (d, J = 8.3 Hz, 2H, Ar–H), 7.48 (d, J = 8.3 Hz, 2H, Ar–H), 5.21 (t, J = 6.2 Hz, 1H, H1), 3.68 (br, 1H, OH), 2.84 (d, J = 6.2 Hz, 2H, H2), 2.21 (s, 3H, CH_3). ^{13}C NMR: δ 208.50 (CO), 148.14, 132.35, 126.35 (Ar-C), 118.73 (CN), 111.29 (Ar–C), 69.05(C4), 51.56 (C3), 30.74 (CH_3).

2.2.2.2 ***4-Hydroxy-4-(4′-nitrophenyl)-2-butanone*** **(2e)** Reaction time: 48 h; elution solvent hexane:ethyl acetate 80:20 v/v, yield: 41%; R_f = 0.17 (hexane/ethyl acetate 70:30). ^{1}H NMR: δ 8.18 (d, J = 8.7 Hz, 2H, Ar–H), 7.54 (d, J = 8.7 Hz, 2H, Ar–H), 5.26 (t, J = 6.3 Hz, 1H, H4), 3,63 (br, 1H, OH), 2.87 (d, J = 6.1 Hz, 2H, H2), 2.22 (s, 3H, CH_3). ^{13}C NMR δ 208.53 (CO), 150.15, 147.27, 126.45, 123.74 (Ar–C), 68.90 (C4), 51.53 (C3), 30.72 (CH_3).

2.2.2.3 ***4-Hydroxy-4-(3′-nitrophenyl)-2-butanone*** **(2f)** Reaction time: 96 h; elution solvent hexane:ethyl acetate 80:20

Table 3 Effect of temperature and BSA mass on BSA-catalysed benzalacetone (**3a**) formation

Entry	Solvent	(BSA/**1a**)[b]	T (°C)	Conversion (%)[a]		
				24 h	48 h	120 h
1	Acetone/ethanol 1:1	5.2	30	15	27	51
2	Acetone/ethanol 1:1	5.2	45	22	47	58
3	Acetone/water 1:1	5.2	30	15	34	54
4	Acetone/water 1:1	5.2	45	46	80	85
5	Acetone/water 1:1	10.4	45	74	96	97
6	Acetone/water 1:1	10.4[c]	45	nd[f]	65	nd
7	Acetone/water 1:1	10.4[d]	45	nd	66	nd
8	Acetone/water 1:1	10.4[e]	45	nd	2	nd
9	Acetone/water 1:1	15.6	45	82	97	98

[a]Determined by HPLC

[b]Biocatalyst/substrate mass ratio

[c]BSA pretreated with urea at 30 °C (as described in Table 1)

[d]BSA pretreated with H_2O at 100 °C

[e]BSA pretreated with urea at 100 °C

[f]*nd* not determined

Table 4 BSA-catalysed aldol reaction of benzaldehydes **1a–f** with acetone

Entry	Substrate	T (°C)	t (h)	Conversion to enone **3a–f** (%)[a]	Conversion to ketol **2a–f** (%)[a]	Enone yield (%)[b]	Cetol yield (%)[b]
1	**1a**	45	48	96	–	93	–
2	**1b**	45	120	96	–	87	–
3	**1c**	45	72	88	–	65	–
4	**1d**	45	72	33	66	25	54
5	**1d**	60	96	63	37	53	25
6	**1d**[c]	45	96	97	–	84	–
7	**1e**	45	48	24	47	18	41
8	**1e**	60	72	32	67	24	58
9	**1e**[c]	45	168	35	53	26	45
10	**1f**	45	96	37	61	34	44

Reactions were carried out in acetone/water 1:1 at a (BSA /**1a–f**) mass ratio = 10.4 (See Sect. 2)

[a]Determined by HPLC

[b]After silicagel column chromatography

[c]In acetone:ethanol 1:1

v/v, yield: 44%; R_f = 0.17 (hexane/ethyl acetate 70:30). ^{1}H NMR δ 8.24 (m, 1H, Ar–H), 8.13 (ddd, J = 8.2 Hz, 2.3 Hz, 1.0 Hz, 1H, Ar-H), 7.72 (m, 1H, Ar–H), 7.53 (t, J = 7.9 Hz, 1H, Ar–H), 5.27 (t, J = 6.2 Hz, 1H, H4), 2.90 (d, J = 6.2 Hz, 2H, H3), 2.24 (s, 3H, CH_3). ^{13}C NMR δ 208.63 (CO), 148.36, 144.96, 131.86, 129.51, 122.55, 120.72 (Ar–C), 68.77 (C4), 51.56 (C3), 30.72 (CH_3).

2.2.2.4 ***(E)-4-Phenyl-3-buten-2-one*** **(3a)** Reaction time: 48 h; elution solvent hexane:ethyl acetate 97:3 v/v, yield: 93%; R_f = 0.57 (hexane/ethyl acetate 70:30). ^{1}H NMR δ 7.56 (m, 3H, 2 Ar-H, H4), 7.42 (m, 3H, Ar–H), 6.75 (d, J = 16.3 Hz, 1H, H3), 2.41 (s, 3H, CH_3). ^{13}C NMR δ 200.22 (CO), 146.97 (C4), 135.88, 129.46, 129.02, 128.06 (Ar–C), 126.52 (C3), 26.78 (CH_3).

2.2.2.5 ***(E)-4-(4′-Methoxyphenyl)-3-buten-2-one*** **(3b)** Reaction time: 120 h; elution solvent dichloromethane, yield: 87%; R_f = 0.50 (hexane/ethyl acetate 70:30). ^{1}H NMR: δ 7.51 (m, 3H, 2 Ar-H, H4), 6.94 (d, J = 8.8 Hz, 2H, Ar–H), 6.63 (d, J = 16.2 Hz, 1H, H3), 3.87 (s, 3H, OCH_3), 2.38 (s, 3H, CH_3). ^{13}C NMR: δ 198.41 (CO), 161.61 (Ar–C), 143.25 (C4), 129.96, 127.06 (Ar–C), 125.03 (C3), 114.44 (Ar-C), 55.41 (OCH_3), 27.41 (CH_3).

2.2.2.6 ***(E)-4-(4′-Chlorophenyl)-3-buten-2-one*** **(3c)** Reaction time: 72 h; elution solvent hexane:ethyl acetate 95:5 v/v, yield: 65%; $R_f = 0.57$ (hexane/ethyl acetate 70:30). ^{1}H NMR: δ 7.47 (m, 3H, 2 Ar–H, H4), 7.37 (d, J = 8.5 Hz, 2H, Ar–H), 6.69 (d, J = 16.3 Hz, 1H, H3), 2.38 (s, 3H, CH_3). ^{13}C NMR: δ 198.06 (CO), 141.86 (C4), 136.43, 132.92, 129.39, 129.26 (Ar–C), 127.48 (C3), 27.69 (CH_3).

2.2.2.7 ***4-[(1E)-3-Oxo-1-buten-1-yl]benzonitrile*** **(3d)** Reaction time: 72 h; elution solvent hexane:ethyl acetate 80:20 v/v, yield: 84%; $R_f = 0.50$ (hexane/ethyl acetate 70:30). ^{1}H NMR: δ 7.71 (d, J = 8.5 Hz, 2H, Ar–H), 7.64 (d, J = 8.2 Hz, 2H, Ar–H), 7.50 (d, J = 16.3 Hz, 1H, H1), 6.80 (d, J = 16.3, 1H, H2), 2.42 (s, 3H, CH_3). ^{13}C NMR: δ 197.62 (CO), 140.63, 138.82, 132.68, 129.79, 128.56 (Ar–C, C3, C4), 118.31 (CN), 113.54 (Ar–C), 27.98 (CH_3).

2.2.2.8 ***(E)-4-(4′-Nitrophenyl)-3-buten-2-one*** **(3e)** Reaction time: 72 h; elution solvent hexane:ethyl acetate 80:20 v/v, yield: 18%; $R_f = 0.50$ (hexane/ethyl acetate 70:30). ^{1}H NMR δ 8.26 (d, J = 8.8 Hz, 2H, Ar–H), 7.71 (d, J = 8.6 HZ, 2H, Ar–H), 7.55 (d, J = 16.3 Hz, 1H, H4), 6.83 (d, J = 16.3, 1H, H3), 2.43 (s, 3H, CH_3).^{13}C NMR: δ 197.53 (CO), 148.58, 140.67, 140.06 (Ar–C, C4), 130.38 (C3), 128.81, 124.20 (Ar–C), 28.05 (CH_3).

2.2.2.9 ***(E)-4-(3′-Nitrophenyl)-3-buten-2-one*** **(3f)** Reaction time: 96 h; elution solvent hexane:ethyl acetate 80:20 v/v, yield: 34%; $R_f = 0.42$ (hexane/ethyl acetate 70:30). ^{1}H NMR δ 8.41 (t, J = 1.9 Hz, 1H, Ar-H), 8.25 (ddd, J = 8.2 Hz, 2.2 Hz, 1.0 Hz, 1H, Ar–H), 7.87 (d, J = 7.7 Hz, 1H, Ar–H), 7.62 (t, J = 8.0 Hz, 1H, Ar–H), 7.56 (d, J = 16.3 Hz, 1H, H4), 6.85 (d, J = 16.3 Hz, 1H, H3), 2.43 (s, 3H, CH_3). ^{13}C NMR δ 197.58 (CO), 148.72 (Ar–C), 140.18 (C4), 136.28, 133.77, 130.06 (Ar–C), 129.39 (C3), 124.69, 122.60 (Ar-C), 28.05 (CH_3).

2.3 BSA-Catalyzed Dehydration of 2e

BSA (54 mg) was added to a solution of 4-hydroxy-4-(4′-nitrophenyl)-2-butanone (**2e**, 0.05 mmol) in acetone/water 1:1 v/v (1 ml) and shaken at 45 °C and 200 rpm for 48 h. Aliquots from the biotransformation were withdrawn at different times and after centrifugation, analysed by HPLC.

2.4 Assays of BSA Reusability

To study the recyclability of BSA, experiments involving two substrates were carried out. In the first assay, BSA (540 mg) was added to a solution of benzaldehyde (**1a**, 0.5 mmol) in acetone/water 1:1 v/v (10 ml) and shaked at 45 °C and 200 rpm for 48 h. The mixture was then filtered off to recover the biocatalyst and an aliquot from the filtrate analysed by HPLC. BSA was washed with acetone (3 × 5 ml), dried and reused in subsequent reaction turns using a similar protocol. In the second assay, BSA (540 mg) was added to a solution of *p*-formylbenzonitrile (**1d**, 0.5 mmol) in acetone/ethanol 1:1 v/v (10 ml) and shaked at 45 °C and 200 rpm for 72 h. To recover BSA for reuse, the same procedure above described was applied.

2.5 Computational Experiments on BSA Structural Analysis and Molecular Docking

The protein data bank (PDB) structure of bovine serum albumin (ID:40r0) complexed with naproxen was taken as reference for the bioinformatic study. The numbers of cavities, as well as their properties, were estimated using Fpocket [20]. The pKa values of the protein ionizable residues were estimated using PROPKA [21] and linked to the cavity residues using our own scripts written in Python. The molecular docking was performed at the assayed pH using Autodock Vina software [22]. The conformations of the ligand–protein complexes obtained with Autodock Vina software were then used to estimate interatomic contacts using RING (Residue Interaction Network Generator, [23]).

3 Results and Discussion

First studies were carried out using benzaldehyde (**1a**, Scheme 1) as model substrate and involved a screening of several experimental parameters. In order to assess results properly, control experiments without BSA and with previously treated BSA were carried out (Table 1). In absence of BSA (Entry 1) no benzalacetone formation was observed, ruling out a non-catalysed aldol condensation; compared with untreated BSA (Entry 2), BSA pretreated with urea at 100 °C (Entry 3) rendered BSA inactive. These control experiments confirm then that BSA promotes benzalacetone formation and indicate that the native form of the protein displays the highest catalytic activity.

The solvent is a key parameter in a biotransformation and, as above stated, a green and cheap reaction medium such as ethanol or water (Table 2) is important when planning a reaction; thus, the solvent effect on benzalacetone (**3a**) production was first studied. It can be seen from Table 2 that ethanol improves the conversion to benzalacetone, the highest value (51%) being attained at 50% ethanol (Entry 5). Results also show that addition of water to acetone also increased appreciably enone conversion except for the highest tested water proportion (Entry 14). It is to point out that all the assayed (co)solvent/acetone ratios involved high molar acetone/benzaldehyde (A/B) ratios favoring the formation of benzalacetone (A/B = 278 at 100% acetone and A/B = 69 at 75% (co)solvent). Moreover, although water is

formed in the dehydration step yielding the enone, decrease in conversion to benzalacetone (10%) at 75% water [water/benzaldehyde (W/B) molar ratio = 850] should not to be explained by equilibrium inhibition due to high water proportion, since the highest conversions (54, 56 and 59%) were also attained at high water proportions (20, 25 and 50% water, W/B = 227–567). Furthermore, benzaldehyde was soluble in all assayed water/acetone ratios. Consequently, the effect of the water content on conversion to benzalacetone should not be related to solubility or stoichiometric reasons but to the impact of water on BSA catalytic activity.

The optimal water contents for maximum product conversion from a given substrate and a given transformation is usually very dependent on the solvent [10, 24]; this has also been reported for biotransformations involving BSA. In the Henry reaction, for instance, high and quantitative conversions to the addition product were reached with nitromethane-water mixtures containing 5–98% water, but no conversion took place in anhydrous nitromethane [25]. In a BSA-catalysed tandem Michael addition and intramolecular cyclization in ethanol [26], high product yields were obtained only at 0, 10 and 20% water, while higher water contents afforded lower yields. In contrast, in a related BSA-catalysed one-pot cyclization, high product yield could only be attained in ethanol/water 3:7, while no conversion was observed in absolute ethanol [27].

In our case, since similar conversions were reached at 20, 25 and 50% water (Table 2), the latter was chosen in order to minimise acetone use (Entry 13), according to the trend in chemo- and chemoenzymatic processes to replace organic solvents by economical and environmentally friendly water [28, 29]. Further experiments were then conducted at acetone–water 1:1.

Regarding the effect of temperature, (Table 3) its impact on benzalacetone production was more marked in the reactions carried out in acetone/water than in acetone/ethanol; in the former medium, a conversion to benzalacetone over 80% could be reached (Entry 4). The influence of BSA mass was also tested: by a two- and threefold increase of the biocatalyst mass, almost quantitative conversions of benzaldehyde to benzalacetone were obtained (Entries 5 and 6).

Aiming at widening the substrate scope of the biotransformation, a set of substituted benzaldehydes (**1b–f**, Scheme 1, Table 4) was tested under the most satisfactory experimental conditions found during the previously discussed screening (**1a**, Entry 1). High conversions in the range 88–97% were obtained for three of the assayed substrates (**1a–c**); the replacement of acetone/water 1:1 by acetone/ethanol 1:1 as the reaction medium changed drastically the product mixture profile of **1d** and allowed high conversions of the enone **3d**. In contrast, nitrobenzaldehydes **1e** and **1f** afforded mixtures of the corresponding intermediate ketol and the enone (Entries 7–10).

Table 5 BSA-mediated dehydration of ketol **2e**

Entry	Biocatalyst	Conversion to **3e** (%)[a]	
		24 h	48 h
1	–	5	3
2	BSA[b]	13	18

[a]Determined by HPLC

[b]Reaction was carried out at 45 °C in acetone/water 1:1 and at a (BSA/**1a**) mass ratio = 10.4 (see Sect. 2)

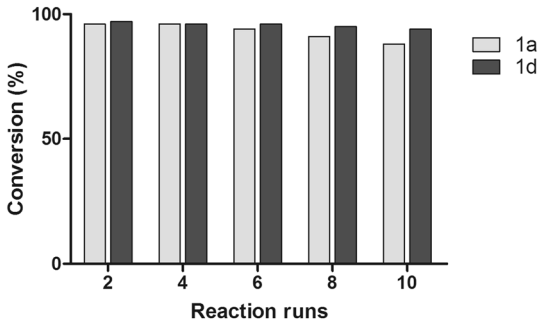

Fig. 1 Reciclability of BSA in aldol condensation of **1a** and **1d**. Reaction runs for **1a** were carried out in acetone/water 1:1 at 45 °C and at a BSA/**1a** mass ratio = 10.4 for 48 h (see Sect. 2); for **1d**, in acetone/ethanol 1:1 at 45 °C and at a BSA/**1d** mass ratio = 10.4 for 72 h. Conversion to enone was determined by HPLC

Scale-up of the biotransformations allowed isolation of the products and their spectroscopic characterization (see Sect. 2) and showed the applicability of this methodology on a preparative scale.

Additionally, in order to know if the dehydration step of the aldol condensation is promoted by BSA, we decided to do some experiments with the ketol **2e** (Table 5). Although BSA activity in some β-elimination reactions has been reported [30, 31], to the best of our knowledge, no previous work has reported experiments studying the role of BSA in the dehydration of β-hydroxycarbonyl compounds. Results in Table 5 suggest that **2e** dehydration giving enone **3e** is promoted by BSA, ruling out a non-catalysed process. It can also be mentioned that chiral gas chromatography analyses of ketols **2d–f** indicated that these compounds were obtained as racemic mixtures.

Finally, the reciclability of the biocatalyst was also considered (Fig. 1), studying the reuse of BSA in the aldol condensation of benzaldehyde (**1a**) and *p*-cyanobenzaldehyde (**1d**) (see Sect. 2). BSA showed good potential for reciclability since in both reactions no significant decrease in conversion to products was observed even after ten consecutive reaction runs.

Focusing now on the catalytic mechanism, the mode of action of BSA is generally assigned to Lys-222, a residue which has an abnormal low pKa and is buried in a pocket, the hydrophobic binding site IIA [17, 32]. The aldol addition has been reported to be accelerated by BSA through an enzyme-like mechanism and inhibited by warfarin, a ligand of the site IIA; moreover, the catalytic activity is conserved in a polypeptide derived from the site IIA sequence. These results enforce the evidence that the observed catalysis is not due to impurities in commercial BSA preparations [18]. In order to further study the structural characteristics that would make possible the catalysis and to explore the structural environment of the binding site IIA, we performed some computational experiments. For that purpose, an open structure of bovine serum albumin (PDB ID:40r0) was taken as reference and using the Fpocket software [33], we explored the biochemical characteristics of albumin pockets. We found a major cavity (having a volume of 2849.9 $Å^3$) showing also the highest polarity and containing an abnormally acidic lysine residue (Lys 221 according to our PDB numeration; pKa = 7.7) and an abnormally acid histidine (His 241 according to our PDB numeration, pKa = 1.37). To the best of our knowledge, no previous works reported the existence of this histidine residue that could be involved in the catalysis, since shifts in acid-base behavior have been observed in residues contributing to the catalytic mechanisms of several enzymes [34]. The mentioned major cavity merges with a smaller one having a volume of 1127.1 $Å^3$, showing the highest hydrophobicity score according to Fpocket and connected to the protein surface through a large tunnel, possibly allowing the transit of substrates between the protein surface and the cavities. Taking into account previous work on BSA catalytic promiscuity, the results herein reported in BSA-catalysed aldol condensation of the series of benzaldehydes **1a–f** and the BSA structural characteristics above mentioned, we performed molecular docking explorations focusing on these cavities. Assayed substrates showed an average of 28 interatomic and Van der Waals contacts with the cavities residues Arg 256, Arg 217, Lys 221, Glu 292 and Arg 199 (see Fig. 2 and Supplementary Material); interestingly, only nitrobenzaldehydes **1e** and **1f** showed absence of contacts with the cavities residues.

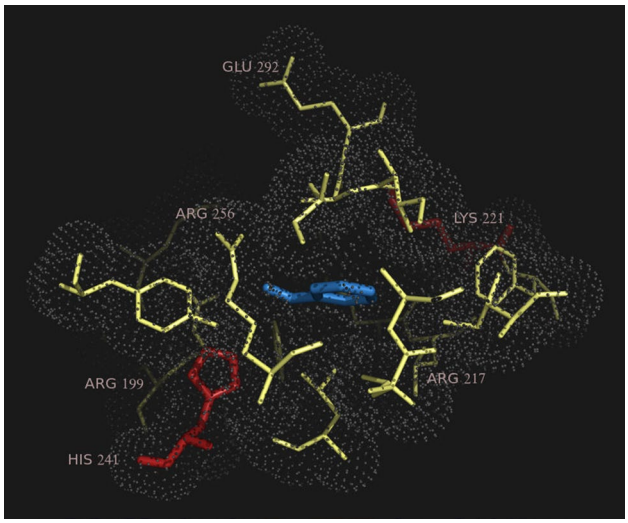

Fig. 2 Benzaldehyde (**1a**; shown in blue) bound to the major cavity of BSA binding site IIA. Cavity residues including Arg 256, Arg 217, Glu 292 and Arg 199 are shown in yellow; abnormally acidic Lys 221 (pKa = 7.7) and abnormally acid His 241 (pKa = 1.37), in red

4 Conclusion

In summary, this work reports a simple and mild procedure to obtain aromatic α,β-unsaturated ketones in good and high yields. Although enone yield is dependent on aldehyde structure, this method applies a green catalyst and water or ethanol, which are cheap and eco-friendly (co)solvents. The mild reaction medium of this procedure contrasts with the reaction conditions of the classic aldol condensation (strong and hot basic media), exhibiting potential for molecules carrying base labile substituents. This method avoids also the use of hazardous or expensive reagents and solvents reported in alternative strategies for the aldol condensation. The potential benefits of an efficient biocatalyst reuse are also remarkable.

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