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Enzymatic Promiscuity: *Escherichia coli* BioH Esterase-catalysed Aldol Reaction and Knoevenagel Reaction

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Abstract Esterase BioH, which is obligatory for biotin synthesis in *Escherichia coli*, was found to exhibit a promiscuous ability to catalyse Aldol and Knoevenagel reactions with moderate to good yields. The reaction conditions including organic solvent, molar ratio of ketone to aldehyde, enzyme amount, and reaction time were investigated to evaluate the effect of different reaction conditions on yield. Target compounds were afforded in the best yield of 91.2% for Aldol reaction and 54.7% for Knoevenagel reaction. In addition, because the enzyme could be prepared with a low cost, this protocol could provide an economic route to conduct Aldol and Knoevenagel reactions, which expand the field of enzymatic promiscuity.

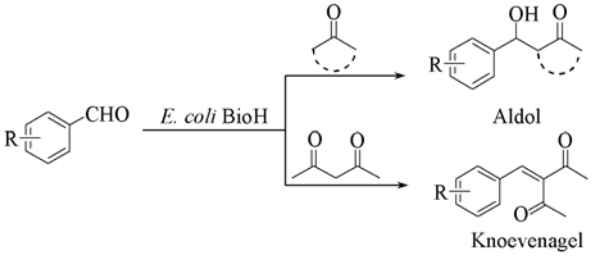
Keywords *Escherichia coli* BioH esterase; Catalytic promiscuity; Aldol reaction; Knoevenagel reaction

1 Introduction

Enzymes have now been generally accepted as valuable catalysts in organic synthesis due to their environmental friendliness, high selectivity and mild reaction conditions[1,2]. Over the past few years, biocatalytic promiscuity, which means to use enzymes to form new bonds and follow new pathways, has attracted significant attention from chemists and biochemists[3]. Some elegant works on this field have been reported in the last ten years. For instance, hydrolases have been used to catalyse Michael addition[4], Markovnikov addition[5], Mannich reaction[6], Morita-Baylis-Hillman reaction[7], Henry reaction, and so on[8]. These encouraged us to believe that enzymatic promiscuity is a common property.

Carbon-carbon bond formation remains a great challenge for organic synthesis. Among different carbon-carbon formation reactions the Aldol reaction and Knoevenagel reaction have received a growing interest as they are atom economic[9,10]. In the past few years, several reports on Aldol reaction and Knoevenagel reaction catalyzed by lipases or protease were published. Wang *et al.*[11] found that PPL, a lipase from porcine pancreas, displayed a promiscuous ability to catalyze the Aldol reaction. A further example was reported by Gotor-Fernándes *et al.*[12] who used protease from *Bacillus licheniformis* as promiscuous catalyst to catalyze the Aldol reaction of acetone to *p*-nitrobenzaldehyde with water as additive at different temperatures. In 2011, Guan and co-workers[13] reported that Nuclease p1 from *Penicillium citrinum* was able to catalyze asymmetric Aldol reaction between aromatic aldehyde and cyclic ketone under solvent-free conditions. After that, they found that papain was able to catalyze the Knoevenagel reaction in DMSO/water[14]. Although significant progress has been made in the field of enzymatic promiscuous catalysis in carbon-carbon formation reactions, there are some drawbacks which need to be overcome, for example, its narrow substrates scope[11], with additives and expensive enzymes[11] used, which is unapplicable in industrial synthesis. Thus, the development of practical biocatalysts is still in demand.

As the part of our ongoing programme on the use of enzymes in the organic syhthesis[15,16], we surprisingly found that *E. coli* BioH(lotus tag b3412 from Gene Bank, EC 3.1.1.85), which naturally catalyzes the synthesis of biotin, also possessed the catalytic promiscuity to catalyze Aldol reaction and Knoevenagel reaction(Scheme 1). The esterase can be easily overexpressed in *E. coli*[17]. Therefore, we believe the low-cost enzyme has potential to realize actual "green" Aldol and Knoevenagel reactions in industry.



Scheme 1 Scheme of *E. coli* BioH esterase-catalyzed Aldol and Knoevenagel reactions

2 Experimental

2.1 Materials and Methods

The NMR spectra were measured on a Bruker Advance 2B 400 MHz instrument with $CDCl_3$ as the solvent and TMS as internal standard. Chemical shifts are expressed in δ relative to the internal standard of tetramethylsilane(TMS). The coupling constants(J) are given in Hz. All the known products were

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characterized by comparing the ^{1}H NMR with those reported in the literature. Reactions were monitored by thin layer chromatography(TLC) with Haiyang GF254 silica gel plates, with a developing reagent consisting of petroleum ether/ethyl acetate (1:1 for Aldol reaction, 3:1 for Knoevenagel reaction, volume ratio). BioH esterase(EC 3.1.1.85) was expressed in *Escherichia coli* and used without further purification[17]. Unless otherwise noted, all the reagents were obtained from commercial suppliers and were used without further purification.

2.2 General Procedure for Aldol Reaction

A mixture of aldehyde(0.5 mmol), anhydrous ketone(7.5 mmol), BioH esterase powder(7.5 mg), cyclohexane(4 mL), water(1 mL) was shaken at 200 r/min at 37 °C for 200 h (formation of product was determined by TLC). The reaction was terminated by filtering off the enzyme. The crude residue was then evaporated in vacuum and purified by silica gel column chromatography with an eluent consisting of petroleum ether/ethyl acetate(2:1, volume ratio). Product-contained fractions were combined, concentrated, and dried.

2.3 General Procedure for Knoevenagel Reaction

A mixture of aldehyde(0.5 mmol), acetylacetone(7.5 mmol), DMF(4 mL), H_2O(1 mL), BioH esterase powder(15 mg) was shaken at 200 r/min at 37 °C for 168 or 200 h(formation of product was determined by TLC). The reaction was terminated by filtering off the enzyme. The crude residue was then evaporated in vacuum and purified by silica gel column chromatography with an eluent consisting of petroleum ether/ethyl acetate(6:1, volume ratio). Product-contained fractions were combined, concentrated, and dried.

2.4 Characterization

4-Hydroxy-4-(4-nitrophenyl)butan-2-one(**3**a)[18]: pale yellow solid, yield: 91.2%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.21(d, *J*=8.8 Hz, 2H), 7.54(d, *J*=8.8 Hz, 2H), 5.27(dd, *J*=8.0, 4.2 Hz, 1H), 3.56(s, 1H), 2.95—2.75(m, 2H), 2.22(s, 3H).

4-Hydroxy-4-(3-nitrophenyl)butan-2-one(**3**b)[18]: pale yellow liquid, yield: 70.3%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.21(d, *J*=8.8 Hz, 2H), 7.54(d, *J*=8.8 Hz, 2H), 5.27(dd, *J*=8.0, 4.2 Hz, 1H), 3.56(s, 1H), 2.95—2.75(m, 2H), 2.22(s, 3H).

4-Hydroxy-4-(2-nitrophenyl)butan-2-one(**3**c)[18]: pale yellow solid, yield: 62.1%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 7.96(d, *J*=8.1 Hz, 1H), 7.89(d, *J*=7.9 Hz, 1H), 7.67(t, *J*=7.5 Hz, 1H), 7.44(t, *J*=7.7 Hz, 1H), 5.67(d, *J*=9.4 Hz, 1H), 3.74(s, 1H), 3.13(d, *J*=17.8 Hz, 1H), 2.70(dt, *J*=34.4, 17.2 Hz, 1H), 2.23(s, 3H).

2-[Hydroxy(4-nitrophenyl)methyl]cyclopentanone(**3**d)[19]: yellow solid, yield: 56.4%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.29—8.08(m, 2H), 7.54(t, *J*=8.0 Hz, 2H), 5.43(d, *J*=2.6 Hz, 1H), 4.86(d, *J*=9.2 Hz, 1H), 2.37(dddd, *J*=40.7, 30.2, 19.7, 9.6 Hz, 3H), 2.07—1.97(m, 1H), 1.83—1.62(m, 2H), 1.62—1.42 (m, 1H).

2-[Hydroxy(3-nitrophenyl)methyl]cyclopentanone(**3**e)[19]: yellow solid, yield: 46.5%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.25(s, 1H), 8.19—8.02(m, 1H), 7.68(dd, *J*=26.5, 16.0 Hz, 1H), 7.54(td, *J*=7.9, 3.9 Hz, 1H), 5.44(d, *J*=2.5 Hz, 1H), 4.84(d, *J*=9.3 Hz, 1H), 2.62—2.22(m, 3H), 2.14—1.91(m, 2H), 1.77(dt, *J*=14.6, 10.0 Hz, 2H), 1.64—1.45(m, 1H).

4-[Hydroxy(2-oxocyclopentyl)methyl]benzonitrile(**3**f)[19]: yellow solid, yield: 35.6%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 7.77—7.59(m, 2H), 7.48(t, *J*=6.7 Hz, 2H), 5.38(d, *J*=2.7 Hz, 1H), 4.79(d, *J*=9.2 Hz, 1H), 2.66—2.19(m, 3H), 2.00(tdd, *J*=20.7, 16.1, 8.3 Hz, 2H), 1.84—1.63(m, 2H).

(*E*)-4-(3-Oxobut-1-enyl)benzonitrile(**4**a)[18]: white crystal, yield: 53.6%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 7.66(dd, *J*=24.3, 8.3 Hz, 4H), 7.49(d, *J*=16.3 Hz, 1H), 6.78(d, *J*=16.3 Hz, 1H), 2.41(s, 3H).

3-(4-Nitrobenzylidene)-pentane-2,4-dione(**6**a)[20]: dark yellow oil, yield: 54.7%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.23(d, *J*=8.5 Hz, 2H), 7.63—7.39(m, 3H), 2.44(s, 3H), 2.27(s, 3H).

3-(3-Nitrobenzylidene)pentane-2,4-dione(**6**b)[20]: yellow oil, yield: 48.1%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.33—8.13(m, 2H), 7.70(d, *J*=7.7 Hz, 1H), 7.58(t, *J*=8.3 Hz, 1H), 7.47(s, 1H), 2.45(s, 3H), 2.30(s, 3H).

3-(2-Nitrobenzylidene)pentane-2,4-dione(**6**c)[20]: yellow oil, yield: 41.6%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 8.21(d, *J*=8.1 Hz, 1H), 7.89(s, 1H), 7.59(tt, *J*=27.5, 13.7 Hz, 2H), 7.37(d, *J*=7.5 Hz, 1H), 2.46(s, 3H), 2.09(s, 3H).

3-(4-Methylbenzylidene)pentane-2,4-dione(**6**d)[20]: pale yellow oil, yield: 35.1%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 7.46(s, 1H), 7.28(dd, *J*=13.7, 11.2 Hz, 2H), 7.19(d, *J*=8.0 Hz, 2H), 2.49—2.39(m, 3H), 2.37(s, 3H), 2.28(d, *J*=13.5 Hz, 3H).

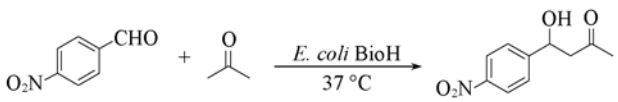
3-(4-Fluorobenzylidene)pentane-2,4-dione(**6**e)[20]: white solid, yield: 43.0%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 7.53—7.37(m, 3H), 7.16—7.07(m, 2H), 2.43(s, 3H), 2.29(d, *J*=13.2 Hz, 3H).

3-(4-Chlorobenzylidene)pentane-2,4-dione(**6**f)[21]: white solid, yield: 40.5%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 7.40(d, *J*=11.3 Hz, 1H), 7.39—7.24(m, 4H), 2.42(s, 3H), 2.27(d, *J*=12.2 Hz, 3H).

3-(2-Chlorobenzylidene)-pentane-2,4-dione(**6**g)[22]: yellow oil, yield: 38.4%. ^{1}H NMR(400 MHz, $CDCl_3$), δ: 7.79(s, 1H), 7.47(d, *J*=8.0 Hz, 1H), 7.42—7.14(m, 3H), 2.47(s, 3H), 2.18(s, 3H).

3 Results and Discussion

Initial studies were undertaken with *p*-nitrobenzaldehyde and acetone as a model Aldol reaction(Scheme 2). The reaction conditions including organic solvent, molar ratio of ketone to aldehyde, enzyme amount, and reaction time were investigated to evaluate the effect of different reaction conditions on yield. The results are shown in Table 1. It was found that under the conditions: molar ratio of ketone/aldehyde, 15:1, cyclohexane as organic solvent, enzyme loading, 7.5 mg, and reaction time,



Scheme 2 Scheme of *E. coli* BioH esterase-catalyzed Aldol reaction between *p*-nitrobenzaldehyde and acetone

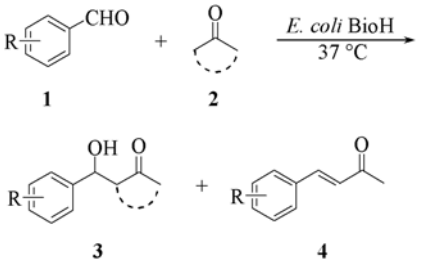
Table 1 Opitimization of the reaction conditions of *p*-nitrobenzaldehyde with acetone*

Entry	*m*(Loading)/mg	Solvent	Molar ratio of acetone to aldehyde	Time/h	Isolated yield(%)
1	—	Cyclohexane	15:1	168	0
2	2.5	Cyclohexane	15:1	168	10.1
3	5	Cyclohexane	15:1	168	46.3
4	7.5	Cyclohexane	15:1	168	89.0
5	10	Cyclohexane	15:1	168	88.3
6	15	Cyclohexane	15:1	168	87.9
7	20	Cyclohexane	15:1	168	88.2
8	30	Cyclohexane	15:1	168	89.1
9	7.5	Dioxane	15:1	168	17.8
10	7.5	THF	15:1	168	15.2
11	7.5	TBME	15:1	168	28.4
12	7.5	DMF	15:1	168	14.7
13	7.5	Acetonitrile	15:1	168	50.4
14	7.5	Solvent-free	15:1	168	41.0
15	7.5	Cyclohexane	5:1	168	34.5
16	7.5	Cyclohexane	10:1	168	84.8
17	7.5	Cyclohexane	20:1	168	87.6
18	7.5	Cyclohexane	25:1	168	82.0
19	7.5	Cyclohexane	30:1	168	86.6
20	7.5	Cyclohexane	15:1	96	65.0
21	7.5	Cyclohexane	15:1	120	79.9
22	7.5	Cyclohexane	15:1	200	91.2

* Reactions were performed on a scale of 5 mL: *p*-nitrobenzaldehyde, 0.5 mmol; acetone; pure enzyme powder of *E. coli* BioH; water content was 20%.

200 h, target compound was afforded in a yield of 91.2%(Entry 22, Table 1).

Subsequently, some other aldehydes and ketones were used to expand upon this *E. coli* BioH-catalyzed Aldol reaction to figure out the generality and scope of its biocatalytic promiscuity(Scheme 3). The results are summarized in Table 2. It was found that a series of *β*-hydroxycarbonyl compounds were obtained as the major products with moderate to good yields(35.6%—91.2%) in all the cases. In the case of aryl aldehydes, the substituent on the phenyl ring of aromatic aldehydes played an important role in the reaction. Nitro-substituted and cyano-substitutde aromatic aldehydes showed moderate to modest yields(Entries 1—7, Table 2). Meanwhile, some substituted aromatic aldehydes(such as *p*-methyl, *p*-fluoro substited benzaldehyde, etc.) were also investigated, however, to our disappointment, no corresponding products were obtained(data not shown). It might be due to the nitro group on the phenyl ring of the aromatic aldehydes was stablilized by the amino acids residues of the *E. coli* BioH *via* hydrogen bond, which was favorable for nucleophilic attack by carbanion. On the other hand, the yields of the *β*-hydroxycarbonyl compound were different due to the position of the nitro group on the phenyl ring of aromatic aldehydes ranging from 62.1% to 91.2%(Entries 1—3, Table 2). Moreover, it is clear that the reactions with acetone were faster than that with cyclopentanone provided the corresponding products with moderate to good yields(Entries 1 and 5, Table 2). This was probably due to the relatively small steric hinderance of methyl. Interestingly, when *p*-cyanobenzaldehyde was used as substrate in this BioH-catalysed Aldol reaction, *α*,*β*-unsaturated carbonyl compound was obtained as the final product due to the intramolecular dehydration(Entry 4, Table 2). Although many substrates and conditions were applied in this reaction to measure the enantioselectivity, there was no obvious optical activity(Entries 1—3, Entries 5—7, Table 2).



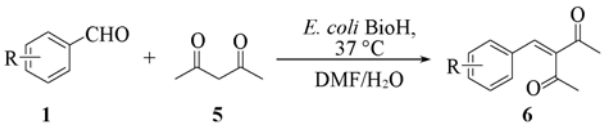
Scheme 3 Scheme of *E. coli* BioH esterase-catalyzed Aldol reaction between aldehydes and ketones

Table 2 *E. coli* BioH-catalyzed Aldol reaction of aldehydes and ketones[a]

Compd.	R	Ketone	Yield[b](%)	*e.e.*(%)	dr(*anti*/*syn*)
3a	*p*-NO_2	Acetone	91.2	13.1	—
3b	*m*-NO_2	Acetone	70.3	5.2	—
3c	*o*-NO_2	Acetone	62.1	3.8	—
4a	*p*-CN	Acetone	53.6	—	—
3d	*p*-NO_2	Cyclopentanone	56.4	12.1	57:43
3e	*m*-NO_2	Cyclopentanone	46.5	7.0	74:26
3f	*p*-CN	Cyclopentanone	35.6	8.6	69:31

a. Experimental conditions: aldehyde(0.5 mmol), ketone(7.5 mmol), pure enzyme powder of *E. coli* BioH(7.5 mg), cyclohexane(4 mL), H_2O (1 mL) at 37 °C, 200 r/min for 200 h; *b*. isolated yields based on aldehyde.

When 1,3-dicarbonyl compounds such as acetylacetone and *p*-nitrobenzaldehyde were used as substrates, we found that BioH also displayed catalytic promiscuity to catalyse the Knoevenagel reaction(Scheme 4) with moderate yields. After optimization, we found a better result with *N*,*N*-dimethyl formamide(DMF) and water as co-solvent. Then we investigated a series of substituted aromatic aldehydes and the results are shown in Table 3. It was observed that aromatic aldehydes bearing either electron-withdrawing group or electron-donating



Scheme 4 Scheme of *E. coli* BioH esterase-catalyzed Knoevenagel reaction

Table 3 *E. coli* BioH-catalyzed Knoevenagel reaction of aldehydes and acetylacetone[a]

Entry	R	Time/h	Compd.	Yield[b](%)
1	*p*-NO_2	168	**6**a	54.7
2	*m*-NO_2	168	**6**b	48.1
3	*o*-NO_2	168	**6**c	41.6
4	*p*-CH_3	200	**6**d	35.1
5	*p*-F	200	**6**e	43.0
6	*p*-Cl	200	**6**f	40.5
7	*o*-Cl	200	**6**g	38.4

a. Experimental conditions: aldehyde(0.5 mmol), acetylacetone(7.5 mmol), pure enzyme powder of *E. coli* BioH(15 mg), DMF(4 mL), H_2O (1 mL) at 37 °C, 200 r/min; *b*. isolated yields based on aldehyde.

group reacted successfully with moderate yields. The aldehyde substituted by an electron-withdrawing group at the *para*-position was converted into the correspongding Knoevenagel product in a higher yield(Entries 1, 5 and 6, Table 3). Meanwhile, ethyl acetoacetate was also investigated, however, no corresponding product was obtained(data not shown).

In conclusion, we described the *E. coli* BioH esterase-catalyzed Aldol reaction and Knoevenagel reaction with moderate to good yields in a wide substrate scope. In addition, because the enzyme could be prepared with a low cost, this protocol could provide an economic route to conduct Aldol and Knoevenagel reactions, which expand the field of enzymatic promiscuity. Further studies on improving its activity and enantioselectivity by enzyme evolution are still in progress in our lab.

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