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PAPER

Mining catalytic promiscuity from *Thermophilic Archaea*: An acyl-peptide releasing enzyme from *Sulfolobus tokodaii* (ST0779) for nitroaldol reactions

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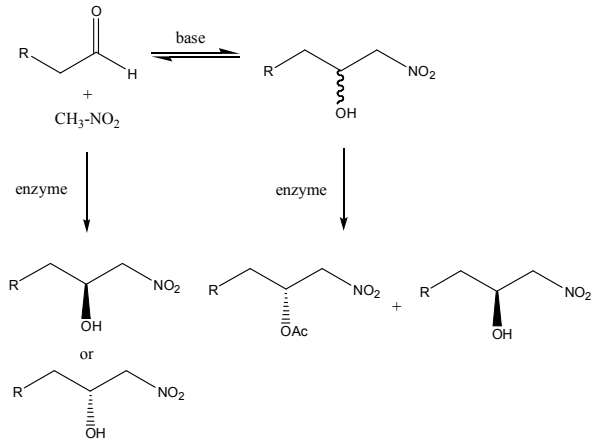
This work demonstrates that the *thermophiles* can be a rich source to mine catalytic promiscuity, whereby an acyl-peptide releasing enzyme from *Sulfolobus tokodaii* (ST0779) is identified to be a promising biocatalyst to mediate Henry (nitroaldol) reaction. Compared to *Porcine Pancreatic Lipase* (PPL), ST0779 displayed superior catalytic efficiency k_{cat}/K_m (6-8 fold higher) and enantioselectivity ee% (90-99%). The catalytic versatility of ST0779 was validated as the enzyme displayed activity towards a broad scope of substituted benzaldehydes; and the electron effects of the benzaldehyde substituents were analyzed by Hammett plotting. Accordingly, this work not only presents a novel enzyme capable to catalyze Henry reaction in higher yield and enantioselectivity than ever reported; but also demonstrates the huge potential of *Thermophilic Archaea* to be an optimal source for mining novel enzymes for biocatalytic promiscuity; which could provide a variety of potent biosynthesis tools to yield diverse kinds of molecules.

Introduction

Enzyme promiscuity is one of the most interesting and potent tools for biosynthesis and organic synthesis.[1-3] It is the ability of an enzyme to carry out alternate reactions following a mechanistic pathway distinctly different from its main reaction.[1-8] To date different classes of enzymes have demonstrated to be capable of yielding various kinds of molecules which is of relevance in the search of synthetic pathways within "Green Chemistry" to obtain compounds of e.g. pharmaceutical interest.[8] However, a distinct disadvantage of the use of enzymes is that they are generally unstable or present poor reusability under the reaction conditions used for synthesis purpose or in industrial processes.[9] Thus, the enzymes from thermophilic and hyperthermophilic organisms which are generally stable at higher temperature and/or in organic solvents, offer a useful source to discover new biocatalysts to overcome this obstacle. In this context, we examine a variety of enzymes from thermophilic bacteria *Thermophilic Archaea* for their catalytic promiscuity with the aim to identify novel enzyme capable to catalyze Henry reaction in high yield and good enantioselectivity.

Henry reaction also known as nitro aldol reaction is one of the most important synthetic tool to form C-C bond.[10-12] This coupling reaction occurs between a nitroalkane bearing α-hydrogens and an electrophilic aldehyde or ketone[13-14] and yields a β-nitroalcohol. This product is typically used to obtain many other intermediates in organic synthesis including aminoalcohols, nitroalkenes, nitroketones. Nitroaldol reactions can be catalyzed by different organic and inorganic bases (i.e. carbonate, hydroxide, alkoxide, or organic nitrogen bases) and promoted by various set of conditions



Scheme 1. Biosynthetic approaches to yield enantio-enriched β-nitroalcohols.

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including the use of aprotic or protic solvents or solvent free systems. However, the use of base generally leads to undesired products because they can also catalyze Cannizzaro reaction and water elimination as well. In addition, they generally yield low enantioselectivity. Therefore, the exploration of an efficient, more enantioselective and environmentally friendly catalyst is necessary for several important synthetic applications.

A recent synthetic strategy to yield enantio-enriched β-nitroalcohol is biocatalytic protocols. So far there are two main enzymatic approaches investigated: direct enzyme catalytic nitroaldol reaction and initial chemical nitroaldol reaction followed by enzymatic resolution of the stereoisomers (Scheme 1).[15] In the latter approach, multiples hydrolases have been evaluated for enzymatic resolution of 1-nitro-2-pentanol including Novozym 435 and *Candida antarctic Lipase* B (CALB), *C. antarctica hydrolase* B, *C. antarctica hydrolase* A, *Rhizopus meihei*, *C. rugosa*, *C. lipolytica* and *Burkholderia cepacia*, where CALB demonstrated to possess the best enantiospecificity.[16,17] On the other hand, in the former approach, different classes of enzymes have proved to catalyze Henry reaction; i.e. hydroxynitrale lyase, glycosidase and lipase, etc.[18-20] However, these biocatalysts generally lead to β-nitroalcohols in good yields but poor enantioselectivity or high enantioselectivity but low yields. Therefore, to discover new enzymes with preferable catalytic promiscuity for nitroaldol reaction is imperative in order to develop more efficient biosynthetic protocols to yield enantio-enriched β-nitro alcohols.

Long-term efforts in our group to exploit novel enzyme catalytic promiscuity have led to the discovery that acylpeptide releasing enzyme (AARE) ORF0779 (ORF, open reading frame) from thermophilic archaea *Sulfolobus tokodaii* can catalyze aldol reactions.[21,22] ST0779 demonstrated a superior efficiency in catalyzing aldol reaction compared to porcine pancreatic lipase (PPL); one of the best enzymes for promiscuously catalyzed aldol condensation.[23,24] Since Henry reactions present a similar mechanism to aldol reactions, in this work, we primarily evaluate the catalytic promiscuity of ST0779 for nitroaldol condensation. More importantly, we screen a variety of hydrolases cloned from thermophilic or hyperthermophilic bacteria, particularly *thermophilic archaea*, whereby the cloned ST0779 displayed superior yields (% Yield= 92) and excellent enantioselectivity (%ee= 94%). This exciting finding motivate us to perform a systematic study, with PPL as a control because this enzyme is readily available from commercial sources and performs better (%yield= 49 and reaction time = 18h) than Pancreatin from *porcin pancreas* (%yield= 41 and reaction time = 48h).[25] Accordingly, reaction were optimized including solvent and temperature, mole ratio of substrates, and reaction time. To envisage versatility of ST0779's catalytic promiscuity, a variety of substituted aromatic aldehydes was examined for Henry reaction; and the electron effect of the substituent on the benzaldehyde ring was evaluated using Hammett equation for both ST0779 and PPL.

Results and discussion

The catalytic promiscuity of hydrolases from different sources (self-

Table 1 Catalytic promiscuity of different enzymes for Henry reaction.[a]

Entry	Catalysts		Time/h	Yield /%[b]	ee/ %[c]
1	Tnap0664	Esterase from *Thermotoga naphthophila* RUK-10	18	78	87
2	Ape1547	Esterase from *Aeropyrum pernix* K1	18	81	89
3	ST1737	Aminopeptidase from *Sulfolobus tokodaii*	18	83	85
4	ST2570	Dehalogenase from *Sulfolobus tokodaii*	18	23	84
5	Novozym 435	Lipase B from Candida antarctica	36	12	90
6	PPL	Lipase from Porcine pancreas	18	49	85
7	ST0779	Acyl-peptide releasing enzyme from *Sulfolobus tokodaii*	18	92	94
8	Denatured PPL[d]		24	<5	-
9	Denatured ST0779[d]		24	<5	-
10	No enzyme		24	Trace	-

[a]All reactions were performed at 40 °C for 18 h using 0.1 mM 4-nitro benzaldehyde, 20 mg of enzyme, and 15 equiv of nitromethane with water content 20 % (water/TBME+water, v/v). [b] The yields were determined by HPLC using VP-ODS column. [c] Enantiomeric excess was determined by HPLC using chiral column. [d] Pre-treated at 110 °C for 24 h.

Table 2 Effect of different solvents on Henry reaction yield[a].

Entry	Solvent	Time/h	Yield /%[b]		ee/%[c]	
			0779	PPL	0779	PPL
1	n-propanol	18	Trace	Trace	-	-
2	DMSO	18	Trace	Trace	-	-
3	DMF	18	Trace	29	-	63
4	THF	18	91	47	92	82
5	TBME	18	92	49	94	85
6	Toluene	18	35	42	85	86
7	Acetonitrile	18	80	34	92	73
8	Cyclohexane	18	44	18	59	53
9	CH_2Cl_2	18	5	6	60	88
10	H_2O	18	65	40	68	72

[a]All reactions were performed at 40 °C for 18 h using 0.1 mM 4-nitro benzaldehyde, 20 mg of enzyme, 15 equiv of nitromethane, and % 20 water content. [b]The yields were determined by HPLC using VP-ODS column. [c]Enantiomeric excess was determined by HPLC using chiral column.

cloned and commercial products) for Henry reaction was primarily evaluated (Table 1). The highest yield (92%) and enantioselectivity (94%) was achieved when ST0779 used as catalyst for nitroaldol condensation of 4-nitrobenzaldehyde and nitromethane (Table 1, entry 7); and the background reactions from either the control (Table 1, entry 10) or denatured ST0779/PPL, which only yielded <5% or traces of β-nitro alcohol after 24 hours (Entry 8-10), were subtracted. Contrarily, ST2570 (a Dehalogenase from *Sulfolobus tokodaii*) and commercial Novozym 435 (CAL-B) however afforded very poor yields,[24] despite their considerably good enantioselectivity (Table 1, entries 4-5); whereas another commercial enzyme PPL obtained a medium yield with relatively good selectivity (Table 1, entry 6). Interestingly but not surprisingly, other hydrolases from *Thermophilic Archaea* Tnap0664, Ape1547 and ST1737, also displayed comparatively high catalytic efficiency (78% to 83%) and excellent enantioselectivity (85% to 89%) (Table 1, entries 1-3). This indicates *Thermophilic Archaea* is a truly rich source to mine catalytic promiscuity. However, it is not our attempt to address the promiscuity of all potential thermophilic hydrolases in this work. We thus chose ST0779 (the best hydrolase from *Thermophilic Archaea*) and PPL to compare their promiscuity for nitroaldol reactions. The reaction time, temperature, solvents, molar ratios, and water content were optimized with respect to their effects on the catalytic efficiency and enantioselectivity. In addition, the catalytic versatility of the enzymes was also evaluated by carrying out the reaction with different substituted benzaldehydes and nitromethane.

Assessing solvent effect on nitroaldol reaction. Organic solvents play an important role in biocatalytic reactions as they can affect the activity and stability of enzyme, and also the solubility of substrate.[28] Accordingly, the biocatalytic efficiency and enantioselectivity of ST0779 and PPL were examined in different solvents (Table 2). As observed in Table 2, tert-butyl methyl ether (TBME; Entry 5) and *tetrahydrofuran* (THF; Entry 4) afforded the highest yields in the reaction catalyzed by ST0779 (Yield= 91-92%) and PPL (Yield= 47-49%). Moreover, ST0779 yields were superior to those previously reported when using hydroxynitrile lyase from *Hevea brasiliensis* (HbHNL; Yield= 77%) as a biocatalyst and TBME as solvent; one of the enzyme that affords the best performance so far reported.[29] It is worthy to point out that, from chemistry point of view, both TBME and THF are categorized into ethers. However, when the reactions are performed in polar organic solvents such as n-propanol, dimethylsulfoxide (DMSO), and N,N-dimethyl formamide (DMF), only traces of products were observed suggesting an activity loss of ST0779 in these solvent media (Entry 1-3). Interestingly, although PPL is inactive in n-propanol and DMSO, the enzyme achieved a moderate yields (% Yield= 29) in DMF; however, the real reason is unclear. In addition, Table 2 also shows that when ST0779 or PPL reactions were carried out in acetonitrile, toluene or water; the enzymes resulted at least 5 times more active than when using dichloromethane (DCM) as solvent (Entry 9). While the biocatalytic reaction for ST0779 carried out in acetonitrile (Entry 7) was 16 times more efficient than the reaction in DCM (Entry 9). It seems that there is not an evident correlation between solvent polarity and catalytic efficiency of the enzyme but bulky, polar, aprotic ethers such as THF and TBME promote a higher yield.

As pointed out by Tawakit and Klibanov, the property of the solvents may markedly alter the enantioselectivity, likely by changing enzyme conformation or affecting the enzyme-substrate interaction during the formation of transition state of the reaction.[30, 31] Indeed, ST0779 obtained excellent enantioselectivity in THF, TBME and acetonitrile (92-94%); whereas the ee% values dropped to no more than 60% for the reactions in cyclohexane and dichloromethane. PPL also achieved considerably good enantioselectivity in THF, TBME and toluene (82-86%, ee%), while surprisingly PPL yield 88% ee% values in dichloromethane. This suggests that the same solvent may yield different impact on the enantioselectivity of different enzymes; which means any generalization with respect to the solvent effect on enantioselectivity of enzyme might be too arbitrary.

Effect of temperature on catalytic efficiency and enantioselectivity. According to Arrhenius theory, temperature can increase the internal energy of the substrate molecules to overcome energy barrier and thus enhance the reaction rate and enzyme kinetics.[32]

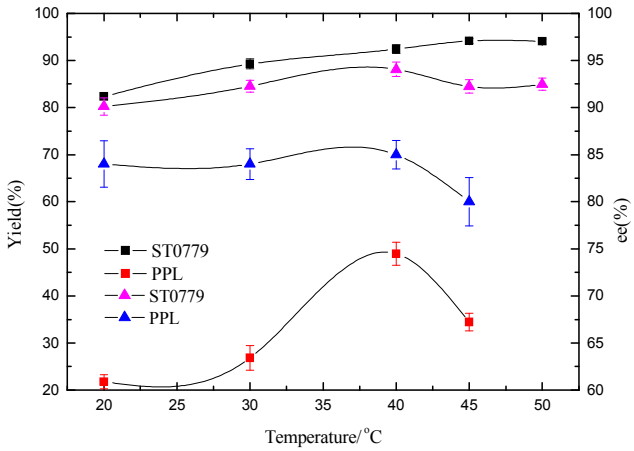


Fig. 1 The effect of temperature on the yield (■, square) and enantiometric excess (▲, triangle) of the Henry reaction. The nitroaldol condensation was carried out at different temperatures for 18 h using 0.1 mM 4-nitro benzaldehyde, 20 mg of enzyme, 15 equiv of nitromethane and water content 20 % (water/ TBME+water, v/v).

Temperature may also affect the enantioselectivity of enzymatic reactions by eliminating the difference in the differential free energy of activation ΔΔG.[32] Therefore, biocatalytic efficiency and enantiomeric excess of the enzymatic reactions were evaluated in different temperature from 20 °C to 50 °C (Fig. 1). Reactions progress was not monitored at higher temperatures because the boiling point of TBME, chosen as a solvent, is 55 °C. As shown in Fig. 1, the reaction of ST0779 affords the desired β-nitroalcohol in good yields at 20 °C (82%) and in excellent yields at 40 °C (90%). Although ST0779 retained a remarkably high ee% (90-94%) from 20 to 50 °C, a slight drop in enantioselectivity when temperature >40 °C is also observed; which could be explained by temperature induced racemization effect.[22] Therefore, ST0779 demonstrates not only to be highly efficient but also yields a pure chiral product. This result differs from other enzymes capable of carrying out henry reactions whereas their catalytic efficiency has to be generally sacrificed in order to afford high enantiomeric excess.[18,29,33] In contrast, the temperature demonstrated a more significant effect for PPL-mediated reaction. As observed in Fig. 1, the yield increases more than two times (%Yield from 22 to 49) when the temperature rises from 20 to 40 °C. However, 45 °C of temperature renders the enzyme deactivation as catalytic efficiency decreases dramatically compared to the reaction at 40 °C. In addition, Fig. 1 also shows that the % ee remains around 70 % up to 40°C but declines when the temperature increases further, indicating that elevated temperature progresses a more significant racemization impact for PPL than for ST0779. In short, ST0779 demonstrates to be a superior biocatalyst to PPL not only in a much higher catalytic efficiency but also in an excellent enantioselectivity over a broad range of temperature, indicating its potential value as a biocatalyst for nitroaldol condensations.

Water content effect on reaction progress. Water plays an important role for enzyme to maintain its catalytic function in organic solvents. The catalytic efficiency of an enzyme may depend on the hydration degree of the enzyme because it helps the enzymes to keep its molecular flexibility and uphold its optimal conformation.[34] The effect of water content in TBME was thus examined for ST0779 and PPL catalyzed Henry reaction. As shown in

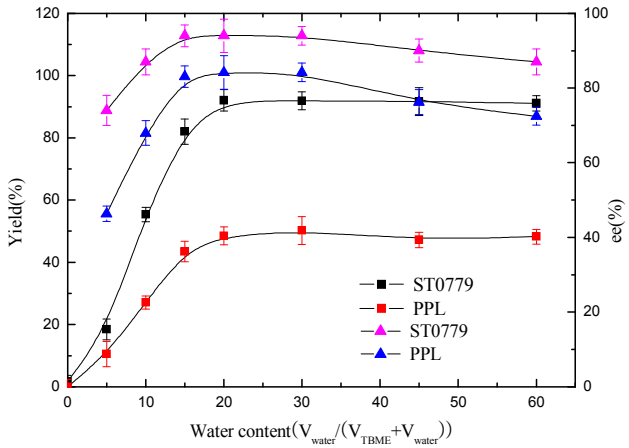


Fig. 2 Influence of Water content on the yield (■, square) and enantiometric excess (▲, triangle) of the Henry reaction. The nitroaldol condensation was carried out at 40 °C for 18 h using 0.1 mM 4-nitro benzaldehyde, 20 mg of enzyme, 15 equiv of nitromethane, TBME as solvent.

Fig. 2, the rate of the Henry reactions increases with water content from 0 to about 20 % (water/TBME+water, v/v) and then levels off thereafter. This suggests that, being neither a reactant nor a product, the role of water in Henry reaction is most likely to be a lubricant to provide enzymes sufficient conformational mobility needed for catalysis.[34] Therefore, once the water content is higher than the threshold; the catalytic reaction becomes independent of water level (Fig. 2). However, the highest %ee resulted when using 15%(v/v) water content in the reaction of ST0779, and 20%(v/v) in the reaction of PPL. Whereas, the %ee for both enzymes decrease smoothly with the increase of water content after 30 %(v/v). This result suggested that the hydration-induced change of conformational flexibility could radically alter catalytic specificity as elsewhere reported.[31,35] We thus can conclude that 20-30% is the optimal water content range for ST0779 and PPL to perform high catalytic efficiency and retain excellent enantioselectivity.

Effect of reaction time on yields and enantioselectivity. The reaction time was optimized using the following coupling conditions: 0.1 mM of 4-nitro benzaldehyde, 15 equiv of nitromethane, 40 °C, 20 mg of enzyme, and 20% and 30% (v/v) of water content for ST0779 and PPL, respectively. As displayed in Fig. 3, the catalytic efficiency of ST0779 was markedly higher than PPL. For instance, after 12 hours ST0779 has afforded 3 times more β-nitroalcohol than when the reaction was run for the same period of time using PPL as biocatalysist. Also, the reaction catalyzed by ST0779 is significantly faster and afford higher yields (Reaction time = 24h; Yield = 90% yield), than the reaction carried out using PPL as biocatalysis (Reaction time = 60h; Yield = 78%). Compared to the Henry reaction catalyzed by HbHNL (77% at 48h),[29] ST0779 demonstrated a remarkably higher efficiency (at least 2 times faster). An interestingly observation is that the enantioselectivities of both ST0779 (%ee, 94%) and PPL (%ee, 84%) are independent of reaction time; which agrees with a previous observation.[29]

Influence of the mole ratio of nitromethane to 4-nitrobenzaldehyde. In essence, nitroaldol reaction is a nucleophilic addition reaction, which nitroalkane is the nucleophile and

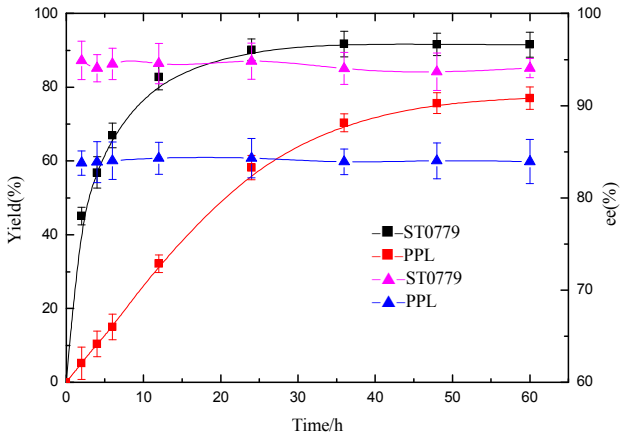


Fig. 3 Time course of yield (■, square) and enantiometric excess (▲, triangle) in henry reaction catalyzed by ST0779 and PPL. The nitroaldol condensation was carried out at 40 °C using 0.1 mM 4-nitro benzaldehyde, 20 mg of enzyme, 15 equiv of nitromethane, and water content 20 % (water/TBME+water, v/v).

substituted aldehyde is the electron-deficient compound. It is therefore logical that excessive dosage of nitromethane favors the shift of the reaction equilibrium to the formation of product thus promotes the conversion of 4-nitrobenzaldehyde.[25,36] Fig. 4 depicts the results (only ST0779 was examined) as expected, that the increase of the mole ratio of nitromethane/4-nitrobenzaldehyde from 1/1 to 10/1 resulted a continuous increase from 27 to about 92 %. This result is in agreement with already reported procedures that typically required around a 10-fold mole excess to perform the biocatalytic Henry reaction and obtain high yields.[36]

Catalytic versatility of ST0779 and PPL. In order to explore the catalytic versatility of both enzymes, different benzaldehydes with electron donating or electron withdrawing substituents were used to perform the Henry reaction. As shown in Table 3, to obtain measurable enantiomeric excess the reaction time varied depending on the aromatic substituent. Shorter reaction time and higher reaction yield were observed for those molecules that present electron withdrawing group as substituents in the aromatic ring. For example, the nitro- and cyanide-group in aromatic aldehydes displayed higher yields and shorter reaction time (Entry 2-4 and 7). While, MeO- which is an electron-donating group that can delocalize benzene ring electron density via p-π conjugation towards the carbonyl functionality, presented a lower yield (Entry 5).

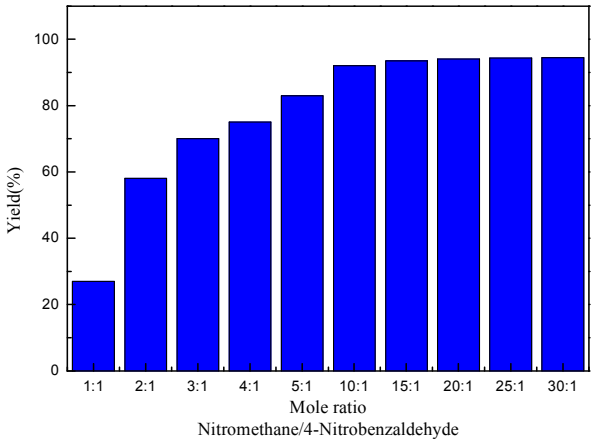
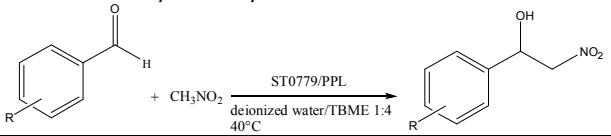


Fig. 4 The effect of mole ratio of nitromethane to 4-nitrobenzaldehyde on the reaction[a]. The nitroaldol condensation was carried out at 40 °C for 18 h using 0.1 mM 4-nitro benzaldehyde, 20 mg of enzyme, and water content 20 % (water/TBME+water, v/v).

Interestingly, for the substituted aromatic aldehydes the reaction enantioselectivity is somehow positively associated with yield, e.g. p-CN, o- NO_2, m-NO_2 and p-NO_2 substituted benzaldehydes achieve 93%-99% enantiomeric excess corresponding to their 89%-92% yield (Entries 2-4 and 7); whereas Entries 1, 5, 6 and 8 give low yield, and from low to moderate ee% values. For all PPL catalyzed reactions, except for 4-cyanobezenzaldehyde, all reactions displayed a low yield (<50%) with varied ee% (Table 3). Nonetheless, the results in Table 3 reveals, both ST0779 and PPL are versatile biocatalysts, which can catalyze Henry reaction of benzaldehydes with varied substituents; furthermore, ST0779 displayed superior properties in both catalytic efficiency and enantioselectivity, which is promising for application in organic synthesis. In addition, compared hydroxynitrile lyase from *Arabidopsis thaliana* (AtHNL) which affords high enantioselectivity (%ee up to 99.9) and shorter reaction times(< 4h),[33] ST0779 does not produce only high enantioselectivity (% ee up to 91) but also higher yields (% yield of ST0779 up to 92 vs %Yield of AtHNL < 34).

To quantitatively characterize the reaction specificity of different substituted aromatic aldehydes with nitroalkane, their apparent rate coefficients were estimated and presented in Table 4. As shown in Table 4, the reaction catalyzed by ST0779 in which 4-nitrobenzaldehyde is the substituent gave the highest apparent rate coefficient (Entry 4). Instead the best reaction coefficient for PPL

Table 3 Biocatalyzed Henry reaction of substituted aromatic aldehydes with nitromethane[a]



		ST0779			PPL		
Entry	R	Time/h[a]	Yield/%[b]	ee/%[c]	Time/h[a]	Yield/%[b]	ee/%[c]
1	H	90	34	17	120	28	43
2	*o*-NO_2	24	90	93	24	39	91
3	*m*-NO_2	24	89	99	24	36	83
4	*p*- NO_2	18	92	94	18	49	85
5	*p*- MeO	72	32	86	120	13	49
6	*p*-Cl	60	45	78	96	26	79
7	*p*-CN	24	92	99	24	71	93
8	*o,p*-diCl	72	35	94	108	33	91

The reaction was performed with 20 mg of enzyme and 20 % water content (water/TBME+water, v/v). [a]The reaction time was set based on monitoring of the reaction by TLC analysis. The resulting products were analyzed by HPLC. [b] The yield were determined using VP-ODS column. [c] Enantiomeric excess was determined using chiral column.

Table 4 Apparent rate coefficients[a] obtained in the reactions catalyzed by ST0779 and PPL

Entry. R	k_{ST0779}[a] min^{-1}	k_{PPL} min^{-1}	Entry R	k_{ST0779} min^{-1}	k_{PPL} min^{-1}
1. H	3.34×10^{-5}	1.98×10^{-5}	5. *p*- MeO	3.88×10^{-5}	9.33×10^{-6}
2. *o*-NO_2	6.94×10^{-4}	1.49×10^{-4}	6. *p*-Cl	7.21×10^{-5}	2.27×10^{-5}
3. *m*-NO_2	6.66×10^{-4}	1.35×10^{-4}	7. *p*-CN	7.62×10^{-4}	3.73×10^{-4}
4. *p*- NO_2	1.02×10^{-3}	2.26×10^{-4}	8. *o*-Cl, *p*-Cl	4.33×10^{-5}	2.68×10^{-5}

[a] $k_{app} = -\log_{10}(A_t/A_0)/t$, t = time. 0.1 mM scale with 20 mg enzyme, 15 equiv of nitromethane in TBME: deionized water 4:1 (v/v) solution at 40 °C. See Table 3 for reaction time.

resulted from the reaction of 4-cyanobenzaldehyde (Entry 7). Not surprisingly, for the reaction of 4-anisaldehyde both enzymes obtained the lowest apparent rate coefficient values (Entry 5).

To further evaluate the substituent polar effect on the reaction rate, Hammett equation was used.[37] The Hammett analysis refers to reaction rates or equilibrium constants of reactions involving derivatives containing a benzyl ring with *meta* and *para* substituents which possess a σ-constant.[38, 39] The σ-constants were derived from the dissociation constant of organic acid, which can frequently be used for reaction rate prediction. In Fig. 5, a linear but not very good correlation of the logarithmic ratios of rate constants $\log(k/k_0)$ with Hammett σ-values were observed for both enzymes.[37] A close inspection of Fig. 5 reveals that for the same substrate ST0779 always achieved $\log(k/k_0) \geq$ that of PPL, which implies a superior catalytic efficiency of ST0779. However, based on the data in Fig. 5 the slope of correlation curve of $\log(k/k_0)$ vs σ-constant was calculated to be 1.33 and 1.30, for ST0779 and PPL, respectively. The comparable values indicate the electron effects of the substituents on reaction rate are independent of the biocatalyst applied; and may also suggest the two enzymes undergo similar catalytic pathway/mechanism.[36]

Kinectic studies for the Henry reactions. To understand the mechanism of the Henry reaction catalyzed by ST0779 and PPL,[40, 41] the kinetic parameters were determined for the nitroaldol condensation between 4-nitro benzaldehyde and nitromethane by varying concentrations of 4-nitrobenzaldehyde with large ratio of excessive nitromethane (15 eqv.) present (Table 5). With respect to the turnover number (k_{cat}) of biocatalyst, ST0779 is always bigger than PPL at the same temperature (2-fold and 1.8-fold at 30 and 40 °C, respectively). As depicted in Table 5, the Michaels constant value (K_m) at both temperatures for PPL were almost 3-4 fold higher than ST0779 catalyzed reaction. This indicates a weak binding/affinity between the active site of PPL and the substrate. In addition, for ST0779, the K_m value declined from 0.72 mM to 0.62 mM as temperature increases from 30 to 40 °C, which suggested an increasing affinity of ST0779 towards substrate at elevated temperature. The k_{cat}/K_m, is a constant to denote the catalytic efficiency of an enzyme widely used to compare the efficacy between different biocatalysts.[42] As shown in Table 5, compared with PPL, ST0779 exhibited a much higher values at both tested temperatures (485.90 vs 57.94 at 30 °C and 731.63 vs 117.13 at 40 °C) ($731.63 M^{-1}s^{-1}$); which means that the catalytic efficiency of ST0779 is 8.4-fold and 6.2-fold higher than PPL at 30 and 40 °C, respectively. Estimated based on Arrhenius equation (Table 5), ST0779 showed a lower activation energy (Ea= 19.84 kJ/mol) compared to PPL, suggesting the temperature can more profoundly alter reaction rate of PPL catalyzed reaction than for ST0779 in nitroaldol condensation. This is different when ST0779 function as a biocatalyst for aldol reaction.[22]

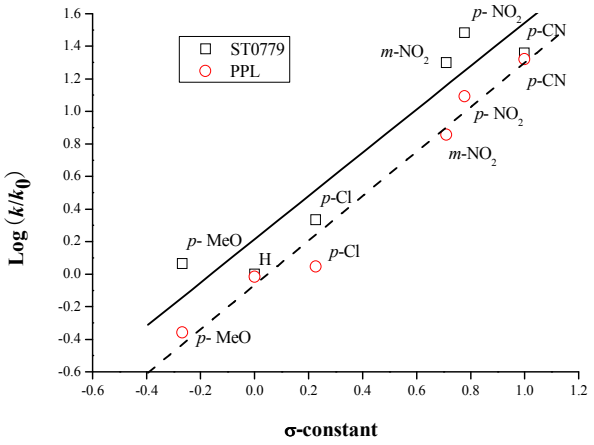


Fig. 5 Hammett plot for the Henry reaction of *meta*- and *para*-substituted benzaldehydes with nitromethane.

Conclusions

In conclusion, this work demonstrated that the thermophiles could be the rich mines to identify new promiscuous enzymes that have high activity and enantioselectivity. ST0779 is found out to be capable of catalyzing nitroaldol reactions in from good to excellent yields with high enantioselectivity; which are much superior to PPL (6-8 fold higher in terms of molecular catalytic efficiency). TBME and THF are found to be the most suitable solvent for ST0779 mediated Henry reaction; and 40°C, 20-30% water content, no less 10:1 of nitrometane/4-nitrobenzaldehyde and around 30h reaction time are the optimal reaction conditions to achieve 90-94% yield and 92-94% enantiomeric excess. Moreover, ST0779 demonstrated a high versatility and it displayed activity towards a broad scope of substrates (substituted benzaldehydes); and the electron effects of the benzaldehyde substituents were analyzed by Hammett plotting. Furthermore, p-CN, and m-NO_2 substituted benzaldehydes

presented the highest yield (92-94%) and enantioselectivity (99%). To our knowledge, compared with other enzymes used to catalyze the Henry reaction reported so far, ST0799 presents the highest combining catalytic efficiency and enantioselectivity in the reaction between 4-nitrobenzaldehyde and nitromethane. This demonstrated the huge potential of *Thermophilic/ hyperthermophilic micr*organism as an exciting and promising source to identify novel catalytic promiscuity; which could contribute to the development of new synthetic approaches and green technology.

Table 5 Estimated kinetic parameters of Henry reaction catalyzed by ST0779 and PPL[a]

Enzyme	Temperature (ºC)	Km (mM)	*k*cat (s^{-1})	*k*cat /Km ($M^{-1}s^{-1}$)	Ea (kJ/mol)
ST0779	30	0.72	0.35	485.90	19.84[b]
	40	0.62	0.45	731.63	
PPL	30	3.07	0.17	57.94	30.44[c]
	40	2.1	0.25	117.13	

[a]Kinetic parameters of the Henry reactions were determined by measuring velocity changes as a function of 4-nitrobenzaldehyde concentration (0.01–0.40 mM) and fitting with Michaelis–Menten equation at the different temperature. [b]Ea of ST0779 was measured at 30–40°C. [c] Ea of PPL was measured at 20–40 °C.

Experimental

Materials All the reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). ST0779, ST1737 and ST2570 were cloned from thermophilic archaea *Sulfolobus tokodaii*. ST0779 and ST1737 expressed in *E. coli* BL21 following the previously described procedures.[21, 26] The enzymes were purified by using Hi-Trap Q-Sepharose column (5 mL, Pharmacia, Uppsala, Sweden) for ST0779 and Ni^{2+}-column affinity chromatography and ion exchange chromatography for ST1737, respectively. ST2570 was cloned in *E. coli* BLP by using vector pET28a and purified by anion exchange chromatography. Ape1547 was cloned from thermophilic archaea *Aeropyrum pernix* K1 and expressed in *E. coli* BL21. The pure protein was obtained by using ion exchange chromatography and gel filtration chromatography following the previously described procedures.[27] Tnap0664 was cloned from the hyperthermophilic bacterium *Thermotoga naphthophila* RUK-10 and expressed in *Escherichia coli* BL21 using pET28a as vector system. The crude protein was purified to homogeneity by anion exchange chromatography. All the proteins power were obtained through lyophilization and stored at -20 °C. PPL in powder form was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Structural identification of Henry reaction synthetic products. All products were characterized through 1H-NMR spectra, and Mass spectroscopy, and High-performance liquid chromatography (HPLC). 1H-NMR were recorded on a Bruker AVANCE III 400 MHz spectrometer using CDCl3 as solvent. MS spectra were measured using a Bruker Maxis Impact electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-QTOF-MS). The melting points of the solid compounds were measured using Differential Scanning Calorimeter on a Pyris 6 DSC system (Perkin-Elmer Cetus, Norwalk, USA). Optical rotations were measured on a polarimeter with a sodium lamp and are reported as $[\alpha]_D^{20}$(c = g/100mL, solvent). The absolute configurations of compounds were assigned by comparison of their optical rotations with literature values.[43-46] HPLC analyses were run on an Agilent 1100 series equipped with an UV detector and an Astec CHIROBIOTICR chiral column to determine enantiometric excess or with a reversed-phase VP-ODS column to determine yield. HPLC analyses were performed using 90 % of Heptane and 10% of Ethanol as eluent. The flow rate and run time varied depending on the column used. When performing analysis to determine yield, the flow rate and run time used were 0.5 mL/min and 20 mins, correspondingly; and when carrying out analysis to determine enantioselectivity, 1 mL/min and 50 mins were used as flow rate and run time, respectively. See the chromatograms of the chiral HPLC separation of Henry reaction products in the Supporting Information.

General procedure for enzyme-catalyzed Henry reactions. In a 25ml glass flask, 20 mg of enzyme, 4 ml of TBME (tert-butyl methyl ether) and 1 ml deionized water (20% v/v water content) containing 4-nitrobenzaldehyde (0.1 mM) and nitromethane (1.5 mmol) were mixed. After 18h stirring at 40 °C, the enzyme was removed by filtration and the reaction mixture was dried over anhydrous Na_2SO_4, filtered, and the remaining solvents were removed under vacuum at 20 mbar and 45 °C. The resulting products were purified using column chromatography and petroleum ether/ethyl acetate (5:2 v/v) as eluent. Reaction progress was monitored by thin-layer chromatography (TLC) and HPLC analysis.

(R)-2-Nitro-1-phenylethanol. Pale yellow oil; ^{1}H-NMR (400 MHz, CDCl3) δ 7.36 – 7.18 (m, 7H), 7.19 – 7.02 (m, 1H), 5.32 (dd, J = 9.6, 3.1 Hz, 1H), 4.65 (dd, J = 7.2, 3.8 Hz, 2H), 4.54 – 4.32 (m, 2H), 2.94 (s, 1H). HR-MS (ESI-IT): exact mass calcd for $C_8H_9NO_3Na$ [M+Na]+: 190.0475m/z, found 190.0472 m/z. % isolation yield= 31 .% ee= 17 (tr=16.9 min(major) and 22.9 min); $[\alpha]_D^{20}$ -3.6 (c 1.5, CH_2Cl_2).

(S)-2-Nitro-1-(2-nitrophenyl)ethanol. Deep red oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.02 (d, J = 8.2 Hz, 1H), 7.89 (d, J = 7.7 Hz, 1H), 7.68 (t, J = 11.2, 4.1 Hz, 1H), 7.49 (t, J = 8.5 Hz, 1H), 5.99 (dd, J = 9.1, 2.1 Hz, 1H), 4.86 – 4.76 (m, 1H), 4.49 (dd, J = 13.9, 9.1 Hz, 1H), 3.12 (s, 1H). HR-MS (ESI-IT): exact mass calcd for $C_8H_8N_2O_5Na$ $[M+Na]^+$: 235.0325m/z, found 235.0324m/z. % isolation yield= 64. %ee=93 (tr=19.2 min and 25.4 min(major)); $[\alpha]_D^{20}$ -230.4 (c 1.1, CH_2Cl_2).

(S)-2-Nitro-1-(3-nitrophenyl)ethanol. White crystal; m.p. 82°C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.32 (s, 1H), 8.22 (dd, J = 8.0 Hz, 1.2 Hz, 1H), 7.77 (d, J = 7.7 Hz, 1H), 7.61 (t, J = 6.0 Hz, 1H), 5.65-5.57 (m, 1H), 4.71 – 4.50 (m, 2H), 3.28 (s, 1H). HR-MS (ESI-IT): exact mass calcd for $C_8H_8N_2O_5Na$ $[M+Na]^+$: 234.2064m/z, found 234.2064m/z. % isolation yield= 63. %ee= 99 (tr=18.0 min and 32.7 min(major)); $[\alpha]_D^{20}$ +35.7 (c 1.5, CH_2Cl_2).

(S)-2-Nitro-1-(4-nitrophenyl)ethanol. Pale yellow crystal; m.p. 82°C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.25 (d, J = 8.5 Hz, 2H), 7.63 (d, J = 8.4 Hz, 2H), 5.71 – 5.49 (m, 1H), 4.68 – 4.49 (m, 2H), 3.27 (s, 1H). HR-MS (ESI-IT): exact mass calcd for $C_8H_8N_2O_5Na$ $[M+Na]^+$: 234.2064m/z, found 234.2065m/z. % isolation yield= 65. %ee= 94 (tr=15.2 min and 25.1 min(major)); $[\alpha]_D^{20}$ +32.4 (c 1.1, CH_2Cl_2).

(S)-1-(2,4-Dichlorophenyl)-2-nitroethanol. Pale yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.50 (d, J = 8.4 Hz, 1H), 7.34 – 7.14 (m, 2H), 5.68 (dd, J = 9.5, 2.2 Hz, 1H), 4.63 – 4.48 (m, 1H), 4.33 (dd, J = 13.5, 9.5 Hz, 1H), 3.43 (s, 1H). HR-MS (ESI-IT): exact mass calcd for $C_8H_7Cl_2NO_3NH_4$ $[M+NH_4]^+$: $C_8H_7Cl_2NO_3H$ $[M+H]^+$: 235.9870m/z,

found 235.9876m/z. % isolation yield= 28. %ee= 94 (tr=9.0 min and 11.7 min(major)); $[\alpha]_D^{20}$ +57.8 (c 1.0, CH_2Cl_2).

(R)-1-(4-Methoxyphenyl)-2-nitroethanol. Pale yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.21 (m, 2H), 6.89 – 6.83 (m, 2H), 5.35 (dd, J = 9.6, 3.0 Hz, 1H), 4.54 (dd, J = 13.2, 9.6 Hz, 1H), 4.41 (dd, J = 13.2, 3.1 Hz, 1H), 3.75 (d, 3H), 2.66 (s, 1H). HR-MS (ESI-IT): exact mass calcd for $C_9H_{11}NO_4Na$ $[M+Na]^+$: 220.0580m/z, found 220.0579m/z. % isolation yield= 38. % isolation yield= 20. %ee= 86 (tr=19.7 min(major) and 25.4 min); $[\alpha]_D^{20}$ -29.2 (c 1.6, CH_2Cl_2).

(S)-1-(4-Chlorophenyl)-2-nitroethanol. Colorless oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.28 (td, J = 8.7, 4.1 Hz, 4H), 5.36 (dd, J = 9.4, 3.1 Hz, 1H), 4.45 (ddd, J = 16.6, 13.4, 6.3 Hz, 2H), 3.01 (s, 1H). HR-MS (ESI-IT): exact mass calcd for $C_8H_8ClNO_3Na$ $[M+Na]^+$: $C_8H_7ClNO_3Na_2$ [M+2Na-H]+: 245.9904m/z, found 245.9913m/z. % isolation yield= 38. %ee=78 (tr=9.6 min and 17.0 min(major)); $[\alpha]_D^{20}$ +21.6 (c 1.1, CH_2Cl_2).

(S)-2-Nitro-1-(4-cyanophenyl)ethanol. White crystal; m.p. 91°C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.64 (d, J = 8.4 Hz, 2H), 7.49 (d, J = 8.1 Hz, 2H), 5.55 – 5.41 (m, 1H), 4.57 – 4.41 (m, 2H), 3.21 (s, 1H). HR-MS (ESI-IT): exact mass calcd for $C_9H_8N_2O_3Na$ $[M+Na]^+$: 215.0427m/z, found 215.0431m/z. % isolation yield= 69. %ee= 99 (tr=18.7 min and 30.5 min(major)); $[\alpha]_D^{20}$ -36.5 (c 1.2, CH_2Cl_2).

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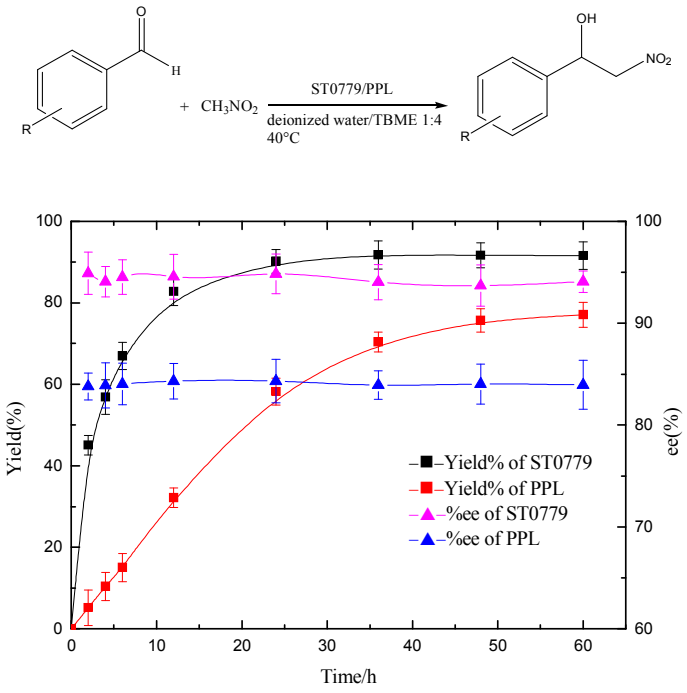
Graphical Abstract



An acyl-peptide releasing enzyme cloned from *Sulfolobus tokodaii* (ST0779) (*Thermophilic Archaea*) displays superior catalytic efficiency k_{cat}/K_m (6-8 fold higher than *Porcine Pancreatic Lipase, PPL*) and excellent enantioselectivity ee% (90-99%); which demonstrated that the *thermophiles* can be a rich source to mine new enzymes for catalytic promiscuity.