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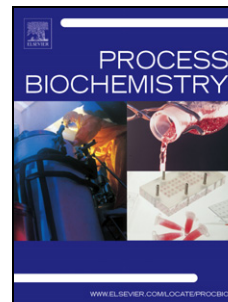

Title: Optimization of chemoenzymatic synthesis of L-arabinose ferulate catalyzed by feruloyl esterases from *Myceliophthora thermophila* in detergentless microemulsions and assessment of its antioxidant and cytotoxicity activities

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Optimization of chemoenzymatic synthesis of L-arabinose ferulate catalyzed by feruloyl esterases from *Myceliophthora thermophila* in detergentless microemulsions and assessment of its antioxidant and cytotoxicity activities

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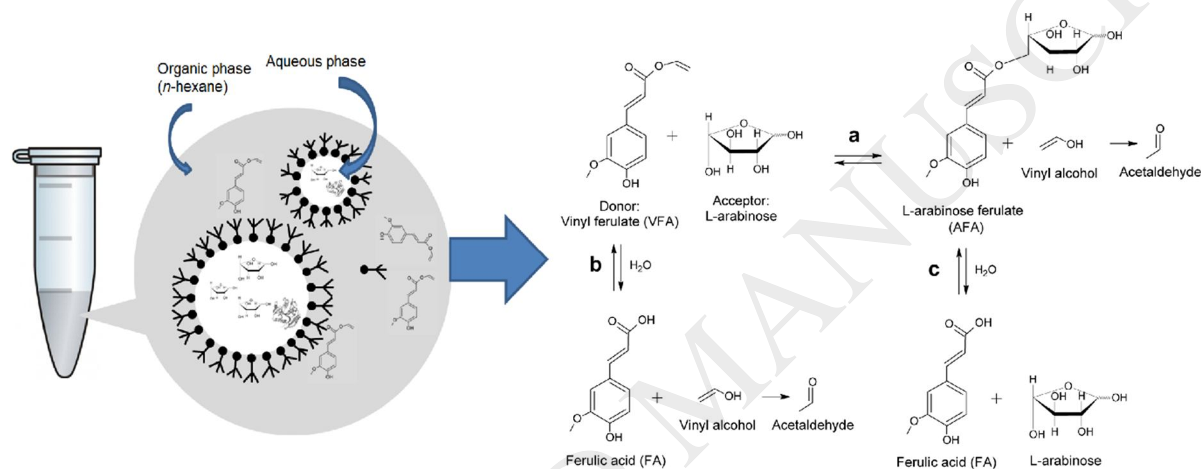

Highlights

- Type A feruloyl esterases (belonging to SF5 phylogenetic subfamily) ~~FAEs~~ from *M. thermophila* C1 have higher affinity for L-arabinose
- FaeA1 offered highest yield (52.2%) at 8 h after optimization of reaction conditions
- FaeB1 offered highest rate (0.602 mol^{-1} g FAE L^{-1} h^{-1}) at 4 h after optimization of reaction conditions
- L-arabinose ferulate ~~AFA~~ is not cytotoxic and has significant scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl ~~DPPH~~ radical

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Abstract

The feruloyl esterases FaeA1, FaeA2, FaeB1, FaeB2 from *Myceliophthora thermophila* C1 and MtFae1a from *M. thermophila* ATCC 42464 were used as biocatalysts for the transesterification of vinyl ferulate (VFA) with L-arabinose in detergentless microemulsions. The effect of parameters such as the microemulsion composition, the substrate concentration, the enzyme load, the pH, the temperature and the agitation was investigated. FaeA1 offered the highest transesterification yield (35.9±2.9%) after 8 h of incubation at 50ºC using 80 mM VFA, 55 mM L-arabinose and 0.02 mg FAE mL^{-1} in a mixture comprising of 19.8: 74.7: 5.5 v/v/v *n*-hexane: *t*-butanol: 100 mM MOPS-NaOH pH 8.0. The ability of L-arabinose ferulate (AFA) to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was significant (IC_{50} 386.5 μM). AFA was not cytotoxic even at high concentrations (1 mM) however was found to be pro-oxidant at concentrations higher than 20 μM when the antioxidant activity was determined with the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay in human skin fibroblasts.

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1. Introduction

Ferulic acid (FA) is the most abundant hydroxycinnamic acid in the plant cell walls with exceptional properties such as antioxidant, antibacterial, antitumor, anti-inflammatory, skin-whitening and UV-absorptive activities, among others [1]-[6]. In graminaceous monocots such as maize, wheat and barley, FA is esterified to the *O*-5 of a-L-arabinose substituents of the xylan backbone, while in few dicots, such as sugar beet and spinach, FA is esterified to the *O*-2 of a-L-arabinose or to the *O*-6 of b-D-galactose in the neutral side chains of pectin [7]. Diferulates occuring in high-arabinose substitutions of arabinoxylan offer cross-linkages with lignin leading to a dramatic increase of mechanical strength of the cells and a decrease of their digestibility by microorganisms [8], [9]. In order to be able to digest the recalcitrant structure of plant cell walls, microorganisms are equipped with a consortium of enzymes such as cellulases, hemicellulases and pectinases.

In particular, feruloyl esterases (FAEs, EC 3.1.1.73) are a subclass of carboxylic acid esterases that are generally known to catalyze the hydrolysis of ester bonds between FA (or other hydroxycinnamic acids) and sugars in plant cell walls. Being very diverse enzymes, FAEs have been classified into four types (A-D) depending on their specificity towards monoferulates and diferulates, for substitutions on the phenolic ring

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and on their amino acid sequence identity [10]. In the recent years, they have received increased attention mainly for their ability to deconstruct plant biomass along with xylanases and other lignocellulolytic enzymes for its utilization in biofuel, paper and pulp and animal feed industry or for the release of hydroxycinnamates that can be used as antioxidants, flavor precursors and functional food additives in the food and pharmaceutical industry [11] - [18]. Less attention has been paid to their synthetic ability as they are able to catalyze the esterification or transesterification of hydroxycinnamic acids and their esters, respectively, with alcohols and sugars under low water content, resulting to products with tailor-made lipophilicity and modified biological properties [19].

Finding the most appropriate biocatalyst for (trans) esterification is a key step. There are numerous reports on the enzymatic acylation of saccharides catalyzed by lipases and proteases [20], [21]. The products have many promising properties such as surfactant, antitumor and plant-growth inhibiting activities; they are amphiphilic, non-toxic and biodegradable [22]. However, lipase-catalyzed esterification of phenolic acids is found to be limited by lower yields due to electronic and/or steric effects revealing that FAEs are attractive biosynthetic tools for hydroxycinnamate modification [23]. The enzymatic acylation of hydroxycinnamic acids with sugars catalyzed by FAEs has been focused on the use of mono- and di-saccharides in detergentless microemulsions resulting in moderate yields (1-60%).

A type C FAE from *Sporotrichum thermophile* (StFaeC) has been used for the transesterification of short chain alkyl ferulates with L-arabinose, D-arabinose and L-arabinobiose reaching a maximum yield of 40%, 45% and 24%, respectively, after 4-5 days when methyl ferulate (MFA) was used as donor [23]-[25]. StFaeC had a broad specificity on saccharides having either a pyranose or furanose ring while it synthesized successfully four linear feruloyl arabino-saccharides, containing from three to six L-arabinose units [26], [27]. The type C FAE from *Talaromyces stipitatus*

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(TsFaeC) catalyzed the conversion of MFA to L-arabinose ferulate at 21.2% yield after 4 days [28]. Direct esterification of FA and transesterification of ethyl ferulate (EFA) with monomeric sugars were carried out using FAE-PL, an enzyme purified from the preparation Pectinase PL "Amano" from *Aspergillus niger* [29]. Various multi-enzymatic preparations containing FAE activity have catalyzed the direct esterification of FA with mono-, di- and oligosaccharides in microemulsions and ionic liquids with maximum yield in the synthesis of feruloyl D-galactose (61%) followed by feruloyl D-arabinose (36.7%) [30], [31].

The potential of FAEs for the synthesis of feruloylated carbohydrates opens the door to design prebiotics and modified biopolymers with altered properties and bioactivities such as delivering phenolics to the colon and thereby reduce the risk of chronic diseases in the distal intestinal region. For instance, the feruloylated oligosaccharides from plant cell walls have shown complementary functional properties, both stimulating the growth of *Bifidobacterium* and protecting against oxidative damage [31]. D-arabinose ferulate was found to be a potential anti-mycobacterial agent with an MIC value against *Mycobacterium bovis* BCG of 25 μg mL^{-1} [24]. On the other hand, the development of competitive synthetic processes for the production of feruloylated sugars, such as L-arabinose ferulate and D-galactose ferulate, could allow further investigation of the natural specificity of FAEs. Until now, the FAE classification is based on synthetic substrates comprising of methyl esters of hydroxycinnamic acids, however in natural environment FAEs cleave the ester bond between FA and arabinose or galactose.

The aim of the present work is the evaluation of five feruloyl esterases (FaeA1, FaeA1, FaeB1, FaeB2 and MtFae1a) from *Myceliophthora thermophila* regarding their ability to synthesize L-arabinose ferulate optimizing various reaction parameters such as the water content, the substrate and enzyme concentration, the pH, the temperature and agitation. The transesterification reaction was performed using the activated

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donor, vinyl ferulate (VFA), and L-arabinose as acceptor, while a competitive side hydrolysis was observed (Fig. 1). *M. thermophila* is a thermophilic filamentous fungus which expresses four FAEs. Four FAEs (FaeA1, FaeA2, FaeB1, FaeB2) have been over-expressed in *M. thermophila* C1 and characterized [32], while MtFae1a, sharing the same sequence with FaeB2, has been heterologically expressed in *Pichia pastoris* and characterized [33]. The five enzymes have been previously optimized for the synthesis of a highly lipophilic feruloyl derivative, prenyl ferulate (PFA) in detergentless microemulsions in analogy with this work [34].

2. Results and discussion

2.1. Hydrolytic and synthetic activity

The FAE activity towards the hydrolysis of VFA and AFA and synthesis of AFA using VFA as donor was determined at fixed conditions (45ºC, 10 min of incubation) (Table 1). It was observed that there is no direct correlation between the hydrolysis in buffer and synthesis in detergentless microemulsions. Although FaeB1 shows highest synthetic activity towards AFA, it has moderate hydrolytic activity towards the same compound. Similarly, FaeB2 has very low synthetic activity while is very robust hydrolytically towards AFA. On the other hand, FaeA1 shows good hydrolytic and synthetic activity. Interestingly, all FAEs showed up to 10-fold increased activity towards AFA comparing to small substituted derivatives of FA such as VFA and MFA, revealing that these enzymes tend to be more robust when active on natural substrates.

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2.2.Effect of medium composition

Transesterification was performed in four different compositions of *n*-hexane: *t*-butanol: 100 mM-NaOH pH 6.0 monitoring the competitive hydrolytic reaction. System IV containing the highest water content and highest concentration in polar *t*-butanol (19.8: 74.7: 5.5 v/v/v *n*-hexane: *t*-butanol: buffer) was considered optimal (Fig. 2). Highest yield (25.9%) and selectivity (0.832) was observed when FaeA1 was used as biocatalyst. Highest rate was observed when FaeB1 was used (0.316 mol g^{-1} FAE L^{-1} h^{-1}).

2.3. Effect of substrate concentration

Under the optimal medium composition, the effect of VFA concentration was examined at fixed conditions. By increasing the VFA concentration, the yield was decreased as expected. The optimal donor concentration, offering highest rate, was applied in subsequent experiments (80 mM for FaeA1, 200 mM for FaeA2, 80 mM for FaeB1, 80 mM for FaeB2 and 150 mM for MtFae1a) (Fig. 3a). Highest yield was 24.5% for FaeA1 at 80 mM while highest rate was observed for FaeB1 at 80 mM (0.372 mol g^{-1} FAE L^{-1} h^{-1}). Selectivity was not increased significantly except in the case of FaeA1 (Fig. 3b).

The effect of L-arabinose concentration was examined at optimal medium composition and VFA concentration. The optimal acceptor concentration offering highest rate, yield and selectivity was equal to 55 mM for FaeA1, FaeA2, FaeB1, FaeB2 and 50 mM for MtFae1a (Fig. 3c, Fig. 3d). Highest yield and selectivity was observed for FaeA1 (30.9% and 1.567, respectively) while highest rate for FaeB1 (0.602 mol g^{-1} FAE L^{-1} h^{-1}). Limiting factors in increasing the acceptor concentration was the insolubility of arabinose in organic solvents and its low solubility in

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water (1 M at 20ºC). In order to overcome the solvent insolubility, L-arabinose was entrapped in the water phase of the microemulsions and was prepared as a stock concentrated solution in buffer containing the enzyme. At concentrations higher than 55 mM, that equals to approximately 1 M of stock solution at 5.5% water (system IV), sugar precipitation was evident after the addition of stock solution into the organic mixture. The solubility limitation of L-arabinose is reflected in the moderate transesterification yields while at concentrations higher than 55 mM, the production of L-arabinose remained constant or was reduced.

The apparent kinetic constants for each substrate were determined by fitting the data on the Michaelis-Menten equation using non-linear regression ($p<0.0001$) (Table 2). Type B FAEs from M. *thermophila* C1 (FaeB2 followed by FaeB1) belonging to SF6 phylogenetic subfamily had the highest affinity towards VFA (lowest K_m) while type A FAEs (FaeA1 and FaeA2) belonging to SF5 subfamily had approximately a 3-fold lower affinity towards VFA. On the other hand, type A FAEs had the highest affinity towards L-arabinose. This finding comes in agreement with by previous reports on substrate specificity profiling showing that type A FAEs have preference on the hydrolysis of more bulky natural substrates, such as feruloyl saccharides rather than small synthetic ones [35]- [38]. FaeB1 catalyzed fastest the transesterification (highest v_{max}) and was the most efficient catalyst for both substrates (highest K_m/k_{cat}). MtFae1a from *M. thermophila* ATCC 42464 had a 4.5-fold lower affinity towards VFA and 1.5-fold higher affinity towards L-arabinose than FaeB2, revealing that glycosylation, a result of production in different hosts, can affect the specificity of an enzyme. Nevertheless, MtFae1a was 128 times less efficient biocatalyst than FaeB2.

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In comparison with our previous work [34], it is observed that in general FAEs synthesize more efficiently the bulky substituted AFA (highest K_m/k_{cat} and v_{max}) than the lipophilic derivative PFA. Moreover, the specificity towards L-arabinose is higher for all tested FAEs independently of the solubility limitations of the acceptor. Finally, the specificity towards VFA is lower when AFA is synthesized, except in the case of FaeB2.

2.4. Effect of enzyme concentration

Enzyme concentration is usually the limiting factor in bioconversion applications. During the synthesis of AFA, enzyme concentration affected yield and selectivity in different manners (Fig. 4). Generally, selectivity (AFA/FA ratio) had an optimum at 2-fold lower enzyme concentrations comparing to the yield. FaeA1, FaeA2 and FaeB2 offered highest yield at 0.02 mg FAE mL^{-1}, while FaeB1 at 0.005 mg FAE mL^{-1} and MtFae1a at 0.1 mg FAE mL^{-1}. On the contrary, the AFA/FA ratio was highest at only 0.005 mg FAE mL^{-1} for FaeA1, FaeB2 and MtFae1a and 0.0002 mg FAE mL^{-1} for FaeA2 and FaeB1 following a linear decrease by the increase in enzyme concentration. The rate decreased exponentially with the addition of more enzyme, as expected (data not known). Highest yield was observed for FaeA1 (30.7%) and highest rate for FaeB1 (0.376 mol g^{-1} FAE L^{-1} h^{-1}).

2.5. Effect of pH

Although the water content of the reaction mixture is low (5.5%), the pH of the aqueous phase in the microemulsion may influence the ionization state of the residues of the enzymes' active site. Optimal yield and rate was observed at pH 8 for FaeA1 and MtFae1a, pH 6 for FaeA2 and FaeB1

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and pH 7 for FaeB2. Optimal selectivity was observed at the same conditions for each enzyme except for FaeB1 (pH 5). Highest yield was 32.8% for FaeA1 while highest rate was 0.398 mol g^{-1} FAE L^{-1} h^{-1} for FaeB1.

2.6. Effect of temperature and time

Transesterification was monitored at different temperatures up to 72 h at optimum conditions for each enzyme (Fig. 5). Among all tested FAEs, FaeA1 had the highest AFA yield (35.9%) at 8 h of incubation at 50ºC. The yield decreased at 31.2% until 24 h and then remained constant up to 72 h. The same profile was observed for selectivity, where the AFA/FA ratio reached a value of 1.120 at 8 h, decreased at 0.592 until 24 h and remained constant thereof. The overall yield was 68.5% at 8 h and increased to 84.7% at 24 h. Accordingly, FaeB1 offered 16.7% AFA yield at 8 h of incubation at 45ºC while it decreased at 15.2% at 24 h. The overall yield was 70.9% and 82.5% at 8 h and 24 h of incubation, respectively, remaining constant thereof. The selectivity increased to 1.100 at 6 h and then decreased to 0.301 and further to 0.225 at 8 and 24 h of incubation remaining constant thereof. As AFA is an amphiphilic compound, we propose that after its synthesis it is transferred partially in the organic phase of the microemulsions that subsequently protects it from further hydrolysis. However, an amount of AFA remains inside the microdroplets where it is further hydrolyzed in small extent until equilibrium is reached, explaining the decrease in transesterification yield with respect to time. FaeB2 and MtFae1a had similar yield (10%) at 30ºC after 24 h of incubation. Lowest yield was observed for FaeA2 (4.5%) after 48 h out of 84.7% overall yield. A summary of the optimal conditions and the obtained parameters is presented in Table 3. Interestingly, at optimal

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conditions the obtained rate comes in agreement with the determined synthetic assay (Table 1), while highest yield is attributed to FaeA1 since the enzyme might not catalyze the reaction with high rate but has high specificity towards L-arabinose.

2.7. Effect of other donors and agitation

The possibility of substituting VFA with other donors (MFA or FA) and the effect of agitation were investigated at optimal conditions for 24 h using FaeA1. As expected, VFA was a more reactive donor while transesterification of MFA was negligible (3.53% at 8 h) (Fig.6a). Direct esterification of FA was not observed. Agitation (1000 rpm) affected negatively the initial rate of reaction (3-fold decrease) while at 24 h the yield was comparable to samples where no agitation was applied (26.13% and 31.2%, respectively). Without agitation, the selectivity reached an optimum at 8 h and then decreased by 50% up to 24 h, while when agitation was applied selectivity increased until 8 h and remained constant up to 24 h (Fig. 6b).

2.8. Cytotoxicity and antioxidant activity

AFA had significant scavenging activity against the DPPH radical with a half maximal inhibitory concentration (IC_{50}) equal to 386.5 μM, assessed after 3 h of incubation while the scavenging yield was found 70% at steady state at concentration 1-4 mM (Fig. 7a). In our previous work [34], the IC_{50} value of FA was estimated to be similar (329.9 μM). Interestingly, AFA was found not to be cytotoxic for normal human skin fibroblasts even at high concentrations (up to 1 mM) (Fig. 7b). Finally, we tested the ability of AFA to reduce intracellular ROS levels with the

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DCFH-DA assay using human skin fibroblasts and found that there was no reduction of basal ROS levels up-to a concentration of 4 μM, while higher concentrations were found to be pro-oxidant (Fig. 7c). The antioxidant activity of phenolic esters is mainly assessed with the DPPH assay where the effect of acylation of the carboxyl group of phenolic acid was been ascribed mainly to the steric hindrance and the H-donating effect of the acyl groups on the interaction with the DPPH free radical and the subsequent resonance stabilization and the formation of quinone [31]. However, there are still some controversies regarding the effect of the molecular structure of phenolic compounds on their reactivity with reactive oxygen species (ROS) in actual living cells. Generally, phenolic carbohydrates are found to be more effective antioxidants towards low-density lipoprotein oxidation than free phenolic acids while isolated feruloylated arabinoxylo-NDOs exhibited a reduced scavenger activity towards DPPH [39]. According to Couto et al. [30], the scavenging yield of D-arabinose ferulate and FA was 70% and 93.1%, respectively, that comes in agreement with our findings, revealing that both enantiomers have similar antioxidant potential. The scavenging activity of feruloylated arabinobiose was equal to the one of FA while the scavenging yield was 83.7% and 92.1% for FA at steady state. The scavenging activity of ferulates towards the DPPH radical seems to be dependent on the chemical structure of the glycosides. The acylation of FA with hexoses (galactobiose, sucrose, lactose, raffinose, and FOS) results in higher scavenging activity as compared with pentoses (arabinobiose, xylobioses and XOS). These results could be explained by the effect of steric hindrance of the glycosidic substituents on the rotation degree of the phenyl moiety [31].

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3. Conclusions

In this work, we evaluated the potential of five FAEs derived from *M. thermophila* to synthesize L-arabinose ferulate in detergentless microemulsions. All tested FAEs preferred a system with higher water content (5.5%) and higher concentration of the polar component (*t*-butanol). Type A FAEs (FaeA1 and FaeA2) belonging to the SF5 subfamily from *M. thermophila* C1 had highest affinity towards L-arabinose but lower affinity towards VFA comparing to Type B FAEs (FaeB1 and FaeB2) belonging to the SF6 subfamily of phylogenetic classification. At optimal conditions FaeA1 had the highest yield (35.9%) at 50ºC, pH 8.0 after 8 h of incubation using 80 mM VFA and 55 mM L-arabinose. Although the hydrolytic and synthetic activity of the tested FAEs is not correlated, it was observed that FaeB1 is an enzyme with unique ability to catalyze the synthesis of AFA at high rates. The efficient chemoenzymatic synthesis of feruloylated sugars such as AFA may open the pathway to the utilization of natural bioactive compounds in amphiphilic preparations, as delivering agents and for the revisitation of classification of FAEs based on natural substrates.

4. Experimental

4.1. Enzymes and materials

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The feruloyl esterases FaeA1, FaeA2, FaeB1 and FaeB2 from *M. thermophila* C1 were over-expressed individually in low-background C1-expression strains while MtFae1a from *M. thermophila* ATCC 42464 was recombinantly expressed in *P. pastoris* strain X33 as reported previously [32],[33],[40]. VFA, as donor for transesterification, and AFA, as standard compound for the assessment of antioxidant and cytotoxicity activities, were provided by Taros Chemicals GmbH & Co. KG (Dortmund, Germany). MFA was purchased from Alfa-Aesar (Karlsruhe, Germany). L-arabinose (≥99%), FA, *n*-hexane (<0.02% water), *t*-butanol (anhydrous, ≥99.5%), MOPS solution 1 M and other materials were purchased from Sigma-Aldrich (Saint Louis, USA).

4.2. Protein and enzyme assays

Protein concentration was determined by the Pierce™ BCA Protein Assay (ThermoFisher Scientific, Waltham, USA). The FAE content (w/w) of the enzymatic preparations was determined by SDS-PAGE and subsequent analysis with the JustTLC software (Sweday, Lund, Sweden). The hydrolytic activity was assayed in 100 mM MOPS-NaOH pH 6.0 at 45ºC for 10 min using 1 mM substrate (AFA, VFA) and different enzyme loads (0.005-1 μg FAE/mL). Reaction was ended by incubation at 100ºC for 5-10 min. The synthetic activity was assayed in 19.8: 74.7: 5.5 v/v/v *n*-hexane: *t*-butanol: 100 mM MOPS-NaOH pH 6.0 at 45ºC for 10 min using 5 mM VFA, 55 mM L-arabinose and different enzyme loads (0.005-0.02 mg FAE/mL). Reaction was ended by addition of acetonitrile. One unit is defined as the amount of enzyme (mg) releasing 1 μmol product per minute under the defined conditions.

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4.3. Transesterification reactions

Enzymatic transesterification was carried out at 500 μL in a ternary system of *n*-hexane: *t*-butanol: buffer forming detergentless microemulsions. Reaction mixtures were prepared by diluting amount of donor in a mixture of *n*-hexane and *t*-butanol followed by vigorous shaking. Reaction was initiated by introducing enzyme and L-arabinose in the form of concentrated stock solution in buffer followed by vigorous shaking until a stable one-phase solution was obtained. Parameters such as the medium composition, the substrate concentration (VFA, L-arabinose), the enzyme concentration, the pH, the temperature and agitation were optimized. Optimal conditions obtained from each study, offering the highest AFA concentration in the mixture, were applied in subsequent experiments.

Unless otherwise stated, reactions were carried out at 50 mM VFA, 30 mM L-arabinose, 0.02 mg FAE/mL FaeA1, FaeA2, 0.002 mg FAE/mL FaeB1, FaeB2, 0.04 mg FAE/mL MtFae1a, 40ºC, 100 mM MOPS-NaOH pH 6.0, 8 h of incubation and no agitation. The effect of medium composition was studied in four different systems (*n*-hexane: *t*-butanol: buffer v/v/v) according to previous reports [26], [24]: system I (37.8: 57.2: 5.0 v/v/v), system II (53.4: 43.4: 3.2 v/v/v), system III (47.2: 50.8: 2.0 v/v/v) and system IV (19.8: 74.7: 5.5 v/v/v). The effect of substrate concentration was studied in a range of 10-300 mM VFA and 10-80 mM L-arabinose while enzyme concentration was varied up to 0.2 mg FAE/mL. The effect of pH was studied using the following buffers (100 mM): sodium acetate (pH 4-6), MOPS-NaOH (pH 6-8) and Tris-HCl (pH 8-10). The effect of temperature was studied at a range of 20-60ºC. The effect of different donors in synthesis was studied using MFA and

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FA. All reactions were prepared in duplicate and accompanied by appropriate blanks while at the end of incubation they were ended by dilution in acetonitrile. No donor consumption (<1%) was observed in the absence of esterase.

4.4.Analysis of feruloyl compounds

Quantitative analysis was made by HPLC on a 100-5 C18 Nucleosil column (250 mm x 4.6 mm) (Macherey Nagel, Düren, Germany) and detection of feruloyl compounds by a PerkinElmer Flexar UV/VIS detector (Waltham, USA) at 300 nm. Samples were diluted 100-fold in acetonitrile before analysis. Elution was done with 70:30 v/v acetonitrile: water for 10 min at flow rate of 0.6 mL/min and room temperature. Retention times for FA, AFA, MFA and VFA were 4.1, 4.4, 6.1 and 7.4 min, respectively. Indication of different anomers was observed by formation of two peaks for AFA, as it is commonly accepted that the main hydroxyl group of (*O*-5) of L-arabinose gets enzymatically transesterified [30], [31], [44]. Calibration curves were prepared using standard solutions of feruloyl compounds in acetonitrile (0.1-2 mM). The sum of molar amounts of the donor and products at the end of reaction was within a 5% error margin compared to the starting molar amount of the donor. The transesterification yield (AFA yield) is defined as the molar amount of generated AFA compared to the initial amount of donor, expressed as a percentage. The overall yield was defined as the molar amounts of AFA and FA compared to the initial amount of donor, expressed as percentage. Product selectivity (AFA/FA molar ratio) was defined as the molar concentration of produced AFA divided by the concentration of produced FA.

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4.5. Isolation of products

In order to estimate the distribution of the feruloyl compounds in the lipophilic and hydrophilic phase of the microemulsions, the physicochemical separation of the system into two phases was attempted. At the end of reaction at optimal conditions using FaeA1, the reaction mixture was diluted fivefold with 100 mM MOPS-NaOH pH 6.0 followed by an equal amount of *n*-hexane. After vigorous shaking, two layers were produced: the upper layer (hexane rich) and the lower phase (water rich). Samples from each layer were withdrawn and analyzed by HPLC. 100% of the unconverted VFA was found in the organic phase while 100% of AFA and FA were found in the lower water phase. Further separation of AFA and FA was done by HPLC and UV detection using an acetonitrile: water linear gradient. Fractions were pooled, evaporated under vacuum and subjected to NMR characterization.

4.6. Structural characterization of L-arabinose ferulate

NMR spectroscopy was performed in DMSO-*d6* with a Bruker Ascend Eon WB 400 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.60 (s, 1H, ArO***H***), 7.57 (d, 1H, *J*= 16 Hz, -C***H***CHCOOR for β-isomer), 7.56 (d, 1H, *J*= 15.6 Hz, -C***H***CHCOOR for α-isomer), 7.34–7.32 (m, 1H, Ar***H***), 7.12 (d, 1H, *J*= 8.3 Hz, Ar***H***), 6.79 (d, 1H, *J*= 8Hz, Ar***H***), 6.49 (d, 1H, *J*= 16 Hz, -CHC***H***COOR for α-isomer), 6.48 (d, 1H, *J*= 15.6 Hz, -CHC***H***COOR for β-isomer) 6.26–6.24 (m, 1H, >OCHO***H***), 5.32–5.28 (m, 2H, >CHO***H***), 5.04 (t, 1H, *J*= 5.3Hz, >OC***H***OH, for β-isomer), 4.95 (q, 1H, *J*= 2.6Hz, >OC***H***OH, for α-isomer), 4.30–4.25 (m, 1H, >CH_2), 4.12–4.08 (m, 1H, >CH_2), 4.00 (dt, 1H, J_1= 2.8Hz, J_2= 6.8Hz, >CH_2C***H***), 3.82 (s, 3H, -OCH_3), 3.74–3.72 (m, 1H, >C***H***OH), 3.68–3.64 (m, 1H, >C***H***OH)

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4.7.Antioxidant activity in a cell-free system

The antioxidant activity of AFA (standard compound) was determined in a cell-free system based on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as described previously [41]. In details, flat-bottomed 96-well plates, serial dilutions of the compound were mixed with an equal volume of 1 mM DPPH in ethanol. Plates were kept in ambient temperature and in dark until measurement of absorbance at 520 nm at various time points. The corresponding dilutions of DMSO served as negative controls. The capacity of the compounds to scavenge free radicals was visualized as reduction of DPPH absorbance.

4.8.Cytotoxicity and antioxidant activity in a cell-based system

Cell-based assays were performed using human skin fibroblasts strain AG01523 (Coriell Institute for Medical Research, Camden, NJ, USA). Briefly, cells were plated in flat-bottomed, tissue culture-treated 96-well plates at a density of 5,000 cells/well in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL) (Biochrom AG, Berlin, Germany) and 15% (v/v) fetal bovine serum (FBS) (Gibco BRL, Invitrogen, Paisley, UK). Cell viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [42]. In details, when cell cultures were confluent the medium was renewed and 18 h later serial dilutions of AFA (standard compound) were added followed by 72 h incubation. Then, the medium was changed to serum-free, phenol red-free

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DMEM containing 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, USA). After incubation with MTT for 4 h, the medium was discarded, and the MTT-formazan crystals were dissolved in 100 μL isopropanol. Cell viability was determined as the reduction of absorbance at 550 nm.

The antioxidant activity, expressed as the capacity for reduction of intracellular levels of reactive oxygen species (ROS) was determined with the 2´,7´-dichlorofluorescein diacetate (DCFH-DA) assay, as described previously [43]. When cultures were confluent, medium was changed to serum-free DMEM and 18 hours later, the serum-free medium was aspirated and renewed with phenol red and serum-free DMEM along with 10 μM DCFH-DA. Following incubation with DCFH-DA for 1 h, serial dilutions of AFA were added and the fluorescence was measured at different time intervals at 480-nm excitation and 530-nm emission. The antioxidant activity of the compounds was visualized as reduction of DCF fluorescence and expressed as % of control. Each experiment was conducted in triplicate.

Author contributions

IA designed, performed the transesterification experiments, analyzed the data and wrote the manuscript; UR, PC designed and analyzed data of the transesterification experiments; AP, DK and MR designed, performed the cytotoxicity and antioxidant activity experiments and analyzed the data; LI produced FaeA1, FaeA2, FaeB1 and FaeB2 from *M. thermophila* C1; GC and VF produced MtFae1a from *M. thermophila* ATCC 42464; PJ and AP synthesized VFA as substrate for the transesterification reactions and AFA as a standard compound for the assessment of antioxidant and cytotoxicity activities; All authors revised the manuscript

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Figure captions

Figure 1 Scheme of a) transesterification of VFA (donor) with L-arabinose (acceptor) b) hydrolysis of VFA (competitive side-reaction) c) Hydrolysis of AFA (competitive side-reaction). Under normal conditions vinyl alcohol tautomerizes to acetaldehyde

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Figure 2 Effect of medium composition on the a) rate and b) selectivity. Reactions were performed in *n*-hexane: *t*-butanol: 100 mM MOPS-NaOH pH 6.0 using 50 mM VFA, 30 mM L-arabinose at 40ºC for 8 h. Black: system I (37.8: 57.2: 5.0 v/v/v), gray: system II (53.4: 43.4: 3.2 v/v/v), striped: system III (47.2: 50.8: 2.0 v/v/v) and white: system IV (19.8: 74.7: 5.5 v/v/v)

Figure 3 Effect of substrate concentration on the a), c) rate and b), d) selectivity. Effect of donor concentration was studied at system IV, 30 mM L-arabinose, 40ºC, 100 mM MOPS-NaOH pH 6.0 for 8 h. Effect of acceptor concentration was studied at system IV and optimal concentration for donor, 40ºC, 100 mM MOPS-NaOH pH 6.0 for 8 h. Black circle: FaeA1, white circle: FaeA2, black square: FaeB1, white square: FaeB2, black triangle: MtFae1a

Figure 4 Effect of enzyme concentration on the a) yield and b) selectivity. Reactions were performed at system IV and optimal substrate concentration for each enzyme, 40ºC, 100 mM MOPS-NaOH pH 6.0 for 8 h. Black circle: FaeA1, white circle: FaeA2, black square: FaeB1, white square: FaeB2, black triangle: MtFae1a

Figure 5 Effect of temperature on the yield. Reactions were carried out at system IV and optimal substrate concentration, enzyme concentration and pH for each enzyme. a) FaeA1 b) FaeA2 c) FaeB1 d) FaeB2 e) MtFae1a

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Figure 6 Effect of donor and agitation on the a) yield and b) selectivity. Reactions were performed by FaeA1 at optimal conditions. Black circle: VFA, no agitation, white circle: VFA, 1000 rpm, black square: FA, no agitation, white square: MFA, no agitation

Figure 7 a) Antioxidant activity determined with the cell-free DPPH assay depending on AFA concentration and time b) Cell viability of human skin fibroblasts assessed with MTT assay following incubation with AFA for 72 h c) Antioxidant activity of AFA determined with the cell-based DCFH-DA assay,

Table 1. Biochemical characteristics of FAEs from *M. thermophila*

Enzyme	Genbank ID	Type	Subfamily[1]	FAE content (mg FAE mg^{-1} protein)	Specific activity (U mg^{-1} FAE)		
					VFA hydrolysis	AFA hydrolysis	AFA synthesis
FaeA1	JF826027.1	A	SF5	0.337	15.80 (2.37)	128.2 (1.9)	1.87 (0.28)
FaeA2	JF826028.1	A	SF5	0.15	17.85 (4.07)	69.5 (8.9)	0.25 (0.01)
FaeB1	API68922.1	B	SF6	0.5	19.28 (6.44)	47.3 (5.2)	4.80 (0.37)
FaeB2	JF826029.1	B	SF6	0.1	19.31 (6.09)	102.1 (6.2)	0.69 (0.06)

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MtFae1a	AEO62008.1	B	SF6	0.422	18.18 (2.53)	57.1 (12.7)	0.67 (0.21)

[1]According to Dilokpimol et al. (2016). Numbers in the parentheses are the estimates of standard deviation

Table 2. Apparent Michaelis-Menten kinetic constants

Enzyme	VFA				L-arabinose			
	v_{max} (mol g^{-1} FAE L^{-1} h^{-1})	K_m (mM)	k_{cat} (10^3 min^{-1} g FAE^{-1})	k_{cat}/K_m (mM^{-1} min^{-1} g FAE^{-1})	v_{max} (mol g^{-1} FAE L^{-1} h^{-1})	K_m (mM)	k_{cat} (10^3 min^{-1} g FAE^{-1})	k_{cat}/K_m (mM^{-1} min^{-1} g FAE^{-1})
FaeA1	0.285 (0.085)	131.2 (59.6)	6.895 (2.056)	52.5 (28.4)	0.194 (0.020)	19.6 (7.1)	4.688 (0.483)	239.7 (90.3)
FaeA2	0.047 (0.006)	153.6 (39.3)	1.404 (0.172)	9.141 (2.6)	0.055 (0.007)	27.8 (8.9)	1.662 (0.212)	59.7 (20.6)

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FaeB1	0.600 (0.079)	56.1 (15.4)	144.9 (19.1)	2582.8 (786.5)	1.129 (0.156)	51.9 (13.5)	272.8 (37.6)	5259.3 (1548.2)
FaeB2	0.312 (0.058)	32.7 (17.6)	85.1 (15.8)	2619.6 (1491.5)	0.431 (0.094)	31.6 (16.3)	118.4 (25.8)	3749.2 (2099.4)
MtFae1a	0.041 (0.008)	148.7 (51.8)	0.661 (0.129)	4.446 (1.775)	0.039 (0.009)	21.4 (15.1)	0.629 (0.145)	29.4 (21.8)

Numbers in the parentheses are the estimates of standard deviation

Table 3. Summary of optimal conditions and obtained parameters

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Enzyme	FaeA1	FaeA2	FaeB1	FaeB2	MtFae1a
Optimized conditions					
Water content (% v/v)	5.5	5.5	5.5	5.5	5.5
VFA concentration (mM)	80	200	80	80	150
L-arabinose concentration (mM)	55	55	55	55	50
Enzyme concentration (g FAE L^{-1})	0.02	0.02	0.005	0.02	0.1
pH	8	6	6	7	8
Temperature (°C)	55	45	45	30	30
Time (h)	8	48	8	24	24
Obtained parameters					
AFA concentration (mM)	28.7 (2.9)	9.0 (0.2)	13.3 (0.9)	7.8 (0.5)	8.7 (1.8)

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AFA yield (% mM AFA/mM ~~VFA~~L-arabinose$_{initial}$)	52.2 (4.3)	16.3 (0.3)	24.3 (1.6)	14.2 (1.0)	17.4 (3.6)
Overall VFA conversion (% mM products/mM VFA$_{initial}$~~%~~)	68.5 (1.8)	84.7 (1.6)	70.9 (5.7)	85.1 (8.8)	52.7 (3.1)
Rate (mol AFA g^{-1} FAE L^{-1} h^{-1})	0.180 (0.015)	0.009 (0.0002)	0.333 (0.021)	0.016 (0.001)	0.004 (0.001)
Initial rate (mol AFA g^{-1} FAE L^{-1} h^{-1})	0.417 (0.026)	0.077 (0.008)	0.602 (0.049)	0.080 (0.012)	0.013 (0.001)
Selectivity (mM AFA/mM FA)	1.120 (0.254)	0.056 (0.0000)	0.308 (0.007)	0.131 (0.005)	0.137 (0.026)

Numbers in the parentheses are the estimates of standard deviation